Summary Report of the Peer Review Meeting: EPA's Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity

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Notice

This report was prepared by Eastern Research Group, Inc. (ERG), a U.S. Environmental Protection Agency (EPA) contractor, as a general record of discussion during the Peer Review Meeting on: EPA's *Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity*, held April 4, 2008, in Arlington, Virginia. This report captures the main points and highlights of the meeting. It is not a complete record of all details discussed, nor does it embellish, interpret, or enlarge upon matters that were incomplete or unclear. Statements represent the individual views of meeting participants.

Contents

1. Introduction

In 2005, the U.S. Environmental Protection Agency (EPA) released the *Guidelines for Cancer Risk Assessment* (EPA/630/P-03-001F) (Cancer Guidelines) and the *Supplemental Guidance Assessing Susceptibility from Early-Life Exposure to Carcinogens* (EPA/630/R-03/003F) (Supplemental Guidance). These documents help EPA risk assessors prepare cancer risk assessments. Specifically, the Cancer Guidelines "provide the framework for determining the mode(s) of action (MOA[s]) by which the chemical induces cancer." The Supplemental Guidance, which supports the Cancer Guidelines, also considers MOAs and recommends the development of separate cancer potencies, based on early- and later-life exposures, for compounds with mutagenic MOAs. These, and other related documents, can be found at www.epa.gov/cancerguidelines.

To expand and clarify the discussions of mutagenic MOAs presented in the Cancer Guidelines and the Supplemental Guidance, EPA developed the *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity* (Framework). EPA prepared the Framework to help EPA risk assessors determine if available data support a mutagenic MOA for carcinogenicity for an agent. Risk assessors are meant to use the Framework in conjunction with the Cancer Guidelines and Supplemental Guidance. Information and approaches presented in the Framework, however, do not supersede those provided in the Cancer Guidelines or the Supplemental Guidance. Rather, the Framework provides a means for organizing data, determining the relevance of these data, and evaluating possible mutagenic MOAs.

Judging that an agent has a mutagenic MOA can have potentially broad impacts. As such, EPA risk assessors must approach the identification of a mutagenic MOA for carcinogenicity in a consistent and scientifically sound manner. In early 2008, Eastern Research Group, Inc. (ERG), an EPA contractor, organized an independent peer review of the External Review Draft of the Framework to assess its scientific quality and utility. Six nationally recognized experts (Appendix A) conducted this review:

- Elaine Faustman, University of Washington
- Robert Heflich, U.S. Food and Drug Administration
- Joseph Landolph, University of Southern California
- Bette Meek (Chair), University of Ottawa
- Jerry Rice, Georgetown University Medical Center
- Toby Rossman, New York University School of Medicine

ERG provided the reviewers with a charge (Appendix B), which asked for their comments on the various aspects of the Framework. Reviewers were also provided with the Cancer Guidelines and Supplemental Guidance. Each reviewer also received complete copies of all written comments submitted during the public comment period, which they were asked to consider.

In the first stage of the review, the experts worked individually to prepare written pre-meeting comments (Appendix C), which were provided to all reviewers and EPA prior to the meeting. In the second stage, ERG convened a one-day peer review meeting, on April 4, 2008, at a facility in Arlington, Virginia. The meeting was attended by 29 observers (Appendix D), including EPA staff and members of the public. Appendix E provides the meeting agenda. The meeting format included an opportunity for public comment (Appendix F). After the meeting, five reviewers submitted post-meeting comments (Appendix G).

This report summarizes the meeting proceedings as follows:

- Sections 2 through 6 provide a detailed summary of the entire meeting. Section 2 presents the opening remarks and Sections 3 through 6 summarize the reviewers' discussions organized by topic area (context and definition of mutagenicity, MOA framework, additional charge questions, and additional comments and issues).
- The appendices provide the following materials: a list of peer reviewers (Appendix A), the charge to peer reviewers (Appendix B), reviewer pre-meeting comments (Appendix C), a list of observers (Appendix D), the meeting agenda (Appendix E), public comments (Appendix F), and reviewer post-meeting comments (Appendix G).

2. Opening Remarks

Jan Connery (ERG), the meeting facilitator, opened the meeting by welcoming the reviewers (Appendix A) and observers (Appendix D), who included the EPA document authors, other EPA staff, and interested members of the public. Connery asked the reviewers and EPA document authors to introduce themselves. Peer reviewers briefly provided background information about their areas of expertise and stated that they had no conflict of interest in reviewing the Framework.

Connery reviewed the meeting agenda (Appendix E). She noted that the pre-meeting comments (Appendix C) were developed by reviewers working individually prior to the meeting. Connery clarified that all meeting discussions would be conducted by the peer reviewers. Reviewers could request, and observers could offer, clarifications where necessary and relevant.

Connery then introduced Lee Hofmann, Executive Director of EPA's Risk Assessment Forum, and Rita Schoeny, Senior Science Advisor for the EPA Office of Water. Schoeny also served as the Chair of the Risk Assessment Forum Technical Panel that developed the draft Framework.

2.1 EPA Remarks

Hofmann briefly welcomed the reviewers, and thanked them for their participation on the peer review panel.

Schoeny provided a brief history of the Framework's development, the processes outlined in the Framework, and the next steps after this peer review meeting. She noted that information provided in her presentation and in the draft Framework does not represent EPA policy.

In March 2005, EPA released the Cancer Guidelines and Supplemental Guidance. Both documents are final and represent EPA science policy. These documents outlined EPA's move from using default assumptions as a first step in cancer risk assessments to using case-specific information and applying default assumptions only if the case-specific information was lacking. MOA became a key factor in cancer risk assessments, with a weight-of-evidence narrative replacing the alphabetic categorization scheme. The Supplemental Guidance focused on differences in cancer risk by life-stage and presented guidance for assessing cancer risk from early-life exposure. The use of age-dependent adjustment factors (ADAFs) for carcinogens acting via a mutagenic MOA was an important component of assessing early-life exposures when adequate data were unavailable for determining chemical-specific life-stage dose-response factors.

During the hazard identification process, an agent's MOA helped to describe the circumstances under which an agent was carcinogenic, such as by what route. The MOA could also inform the relevance of laboratory data to humans. The MOA information also determined the extrapolation method used to move from high to low doses; that is, choice of linear or non-linear extrapolation. Generally, if an agent acted by a mutagenic MOA, then linear low-dose extrapolation would be chosen. If an agent acted by a mutagenic MOA, but the chemical-specific data were insufficient

to develop agent-specific ADAFs, the Supplemental Guidance called for use of default values. These values included a tenfold adjustment to a cancer slope factor when assessing risk for children 2 years old and younger, a default threefold adjustment to the slope factor when assessing risk for children age 2 to 15, and no ADAF for children 16 years and older, including adults. The adjusted slope factors are to be used with life-stage specific exposure information in the risk characterization phase of risk assessment.

Determining whether or not an agent acted as a carcinogen based on a mutagenic MOA then became key in assessing that agent. Accordingly, the EPA Risk Assessment Forum panel (which consisted of EPA scientists) developed the Framework to assist risk assessors in making this determination. These scientists gathered information for the Framework from discussions with peers, literature reviews, case studies, and presentations at scientific meetings. A draft Framework based on this information underwent an internal EPA review in May 2006. After revision, the Framework underwent an inter-agency review, which was facilitated by the Office of Management and Budget, from July 2006 through September 2007. EPA addressed comments and revised the Framework for the public comment period, which closed in December 2007. The external peer review meeting described in this report served as a part of the external peer review process.

Once the Framework is finalized, EPA intends it to serve as EPA science policy, which means that the Framework will be used as guidance by EPA risk assessors in conducting cancer risk assessments. The Framework does not attempt to describe all possible uses of genotoxicity data or mode of action analyses. To minimize possible confusion regarding the use of MOA data in this Framework and its use for other purposes, EPA included a section (1.2) describing the ways this type of data is used at EPA and at other government agencies.

As EPA, does not have an Agency-wide standard definition of "mutagenic," the authors developed an operational definition for use in the Framework. This definition was intended to be consistent with the Cancer Guidelines, Supplemental Guidance, and other relevant guidelines:

> ". . . capacity of either the carcinogen or its metabolite to react with or bind to DNA in a manner that causes mutations. In this context, mutagens usually (though not always) produce positive effects in multiple test systems for different genetic endpoints, particularly gene mutations and structural chromosome aberrations, both *in vitro* and *in vivo*."

Schoeny emphasized that, although the Framework defined "mutagenic," it did not define mutation. Instead, the Framework provided examples of mutations. The document also did not attempt to define differences between the terms "genotoxic" and "mutagenic."

The Framework outlined a multi-step process for assembling, characterizing, and evaluating data to judge whether or not an agent has a mutagenic MOA. This process required that the demonstration of a mutagenic MOA be as scientifically rigorous as the process for any other MOA. Schoeny noted that the document assumed no default MOA; instead the Cancer Guidelines provided default procedures (*i.e.,* linear low-dose extrapolation) for use when a risk assessor could not determine an agent's MOA. Schoeny also recognized that the figures within

the Framework presented the multi-step process as linear, whereas in practice, this process was iterative.

To facilitate the first step in the process—assembling data—the Framework provided sample tables for organizing the data and evaluating the data against acceptance and quality criteria. Schoeny stated that risk assessors were directed to retain all data and include comments regarding data quality rather than eliminate data from consideration. EPA recognized that no one single test or assay could determine whether or not an agent acted through a mutagenic MOA. The results from each of these tests and assays lent differing weights of evidence for or against a mutagenic MOA. The Framework provided some examples of how results from several assays applied to the mutagenic MOA.

To characterize the data, the Framework suggested developing consistent conclusions—positive, negative, inconclusive, and contradictory—for different assay types (*e.g.,* salmonella) and different effects (*e.g.,* point mutation). Schoeny emphasized that the Framework provided no minimum data set requirements or data checklists for drawing a final conclusion that an agent does or does not have a mutagenic MOA. The Framework provided a means for organizing and characterizing available data to allow for a weight-of-evidence analysis in a consistent and transparent fashion.

To make a judgment about the MOA, risk assessors must consider the key events that lead to a cancer endpoint. The Framework stated: "For a chemical to act by a mutagenic MOA, either the chemical or its direct metabolite is the agent inducing the mutations that initiate cancer." Mutagenicity combined with carcinogenicity did not necessarily equal a mutagenic MOA. The Cancer Guidelines provided a list of key events that may implicate mutagenicity as the MOA. They included: early tumor response, initiating activity, mutation in absence of cytotoxicity, and mutation in oncogenes. Using their determination about whether or not a specific chemical is mutagenic, EPA risk assessors will use the procedures discussed in the draft Framework to determine whether the mutagenicity is a key event in causing cancer to develop. Schoeny noted several times that the Framework was developed based on the MOA guidance provided in the Cancer Guidelines and Supplemental Guidanc.

Currently, EPA is reviewing and addressing public comments submitted by 14 parties. EPA condensed their comments into 60 different topic areas, such as definition of mutagenic, multiple MOA, and choice of flow charts (Figure 1, pages 11-13 of the Framework). EPA will respond to each of these comments. EPA was also preparing case studies for cyclophosphamide, coke oven emissions, and very likely several more agents. After the peer review, EPA will respond to the public comments and the peer review comments, and revise the Framework. The revised Framework will undergo clearance through the EPA Risk Assessment Forum and Science Policy Council.

At the conclusion of Schoeny's presentation, the peer reviewers asked several questions. One asked how EPA selected the agents for the case studies. Schoeny responded that cyclophosphamide has a large database, so this agent provided an example of an ideal data set for determining MOA. Coke oven emissions represented a group of chemicals already identified as having a mutagenic MOA. The reviewer noted that case examples with poor or limited data

sets would be more representative of common, real-world situations faced by risk assessors. During later discussions, the reviewers further addressed the need for case examples to support the Framework.

Reviewers asked about the definition of mutation. One reviewer felt that the definition restricted the Framework to agents that directly react with DNA to cause heritable, somatic cell DNA damage. If so, the Framework would not apply to non-chemical carcinogens, such as radiation. Another asked if the mutations must be heritable for a mutagenic MOA. Schoeny responded that the definition was not intended to restrict the Framework to agents that form DNA adducts. Rather, the Framework included all agents that interacted with DNA; it would apply to ultraviolet radiation, for example.

A reviewer asked how an agent that acted as an oxygen radical generator, or agents with dual MOAs, would be handled following the Framework. Schoeny stated that the existing Cancer Guidelines and Supplemental Guidance asked risk assessors to evaluate all possible MOAs. If the available data established that the mutagenic MOA occurred as an early, key event resulting in a cancer endpoint, then the low-dose extrapolations and ADAFs would apply. Schoeny also noted that the Framework was purposely left open-ended to allow use over a wide range of agents.

One reviewer asked why the ADAFs applied only to mutagenic MOAs. Schoeny responded that the mutagenic MOA has shown the most robust relationship between early life exposure and increased cancer susceptibility. She also noted that the science policy articulated in the Supplemental Guidance calls for application of ADAF only when a mutagenic MOA is established, but insufficient data exist to determine life-stage specific slope factors.

2.2 Public Comments

Connery opened the meeting to observer comments. Two observers provided comments: James Swenberg, University of North Carolina, and Craig Barrow, Dow Chemical Company. Appendix F provides the full text of Swenberg's oral comments and the presentation slides from Barrow's oral comments.

Swenberg noted that his expenses for attending this meeting had been paid by the American Chemical Council (ACC); however, he had not shared his opinions or comments with others before this meeting. He agreed that strong scientific consensus existed regarding mutations as a key event in the initiation and progression of cancer. He felt that the Framework provided an opportunity to enhance the application of this knowledge in risk assessment. To that end, he provided the following comments on the Framework.

- The Framework did not clearly differentiate between "mutagenic" and "genotoxicity." He felt that these were two distinct terms and the Framework's use of the terms interchangeably was incorrect.
- DNA adducts represented biomarkers of exposure, and their presence would decline to zero as dose declined. Swenberg provided his publication detailing issues regarding biomarkers in toxicology. (Swenberg, JA; Fryar-Tita, E; Jeong, Y; Boysen, G; Starr, T;

Walker, VE; Albertini, RJ. [(2007)] Biomarkers in toxicology and risk assessment: informing critical dose-response relationships. Chem Res Toxicol 21:253-265)

- In contrast to DNA adducts, mutations had a background incidence that did not approach zero. Agent-induced mutations may have linear or non-linear dose-response curves that extended to within background incidences.
- The Framework did not address the differences in the dose-response curves between DNA adducts and mutations. The dose-response curve for mutations applied to a low-dose, cancer risk assessment, and not the dose-response curve for DNA adducts.
- Most mutagenicity and genotoxicity data were collected as part of the hazard identification process. The Framework should include a discussion of how these data related to key events occurring in cancer bioassays.

Barrow presented comments on behalf of ACC. ACC supported EPA's efforts in improving guidance to keep pace with scientific advancements. Barrow specifically referenced EPA's efforts to reflect the scientific community's greater understanding of chemical carcinogenesis, the applicability and concerns associated with animal models in relation to humans, and promoting MOA in risk assessments across EPA programs. He noted that ACC also understood that EPA must ensure that guidance documents were consistent across the programs and offices. As such, ACC commended EPA's initiative to further the use of mutagenic MOA in risk assessments. However, a single study showing a mutation was insufficient to conclude that an agent acted by mutagenic MOA for cancer endpoints, as noted by EPA.

In this context, Barrow offered several suggestions for improving the Framework:

- The Framework should provide specific guidance that described the steps for organizing data, evaluating data relevance, and weighing the data to render a judgment about the presence of a mutagenic MOA for cancer.
- Both versions of Figure 1 (pages 11-13 of the Framework) were lacking, with version 1 providing more flexibility in applying the Framework guidance. Regardless, the figure needed to include an agent lacking a mutagenic MOA and needed to distinguish between agents with a mutation as a key event versus a mutation that was secondary in tumorigenesis.
- EPA should develop case studies with actual data sets and hold workshops in which participants apply the Framework. These studies would assist in defining the steps and procedures that risk assessors would apply when interpreting real-world data and assessing possible mutagenic MOAs for cancer. As a reviewer of the Framework, he found assessing the application of the guidance to be difficult without case studies.
- The extrapolation from high-dose animal data to low-dose, environmentally relevant exposures also needed careful consideration.
- The Framework should also provide specific and objective guidance on evaluating and assessing both individual study results and the full body of evidence as a whole. This additional guidance would include processes that result in scientifically robust determinations regarding the presence of a mutagenic MOA for cancer.

2.3 Reviewer Discussions

Connery then turned the meeting over to Bette Meek, the panel chair, to begin the reviewer discussions. Prior to the meeting, Meek and Connery developed an agenda that organized the charge questions by topic area, rather than numerically as the questions appeared in the charge to peer reviewers (Appendix B). The reviewers discussed the charge questions by topic area (context and definition of mutagenicity, MOA framework, additional charge questions, and additional comments and concerns). Sections 3 through 6 of this report summarize those discussions. Each of these sections opens with a list of the charge questions discussed under the topic area.

3. Reviewer Discussion: Context and Definition of Mutagenicity

- ***Is the document's purpose clear?***
- ***Is the definition of mutagenicity useful and appropriate, considering the limited application in this Framework?***

The reviewers varied in their opinions about whether or not the Framework's purpose was clearly stated. One reviewer initially commented that the purpose was clear, although with limited application. After reading the written comments from the other reviewers and their interpretations of the Framework, however, this reviewer felt that maybe he was mistaken and the purpose could be more clearly stated. He noted that the Framework was meant to determine if a mutagenic MOA was present, not to identify the specific MOA if it was not a mutagenic one. One reviewer thought that the written comments from Rice best framed the panel's overall thoughts and concerns regarding the document purpose and objectives. This reviewer was unclear about the document objectives and suggested that clarity could be gained if EPA included text that described the process by which they prepared the Framework.

In considering the definition of mutagenicity, reviewers agreed that definition presented in the Framework was too narrow. At the conclusion of their discussions regarding the definition of mutagenicity, they agreed that EPA should broaden and re-frame the definition of mutagenicity with consideration of concerns raised during their discussions, as detailed below, and in their written pre-meeting comments (Appendix C).

Considerations and concerns raised by individual reviewers included the following:

- Properly defining mutagenicity was critical to the processes outlined in the Framework, thought one reviewer. Without a good definition, users could not move forward in applying the Framework. Another reviewer agreed that application of the Framework would fail if the current definition of mutagenicity remained.
- The Framework relied on a definition of mutagenicity that would require risk assessors to consult several documents to fully understand the definition. One reviewer felt that the Framework should be written in a complete fashion, so that it could stand alone. Another reviewer felt that consultation with multiple documents would lead to confusion. This reviewer also noted that the definition of mutagenicity presented in the Framework was inconsistent with definitions used by other EPA offices, U.S. agencies, and international organization.
- EPA began the Framework with a good discussion of the complexities of defining mutagenicity within EPA. Then EPA presented a narrow definition for application of the Framework. This reviewer thought that the discussion of the complexity of defining mutagenicity supported a broader definition within the Framework.
- Reviewers felt that the narrow definition of mutagenicity excluded some causes of DNA mutation. One specifically thought that the definition would include some mutagens that

did not interact covalently with DNA (*e.g.,* intercalating agents), and exclude some agents that do interact covalently with DNA, albeit indirectly (*e.g.,* oxidative mutagens). For example, scientists vary in their opinions about whether or not aneuploidy would be considered a mutation. Many causes of aneuploidy do not involve direct DNA interactions, but rather reactions with proteins; yet some aneuploidy fits the commonly accepted definition of a mutation. Some scientists, however, would argue that aneuploidy was not a mutation, noted one reviewer. Another reviewer argued that adding or subtracting DNA (e.g., additions, gene amplification, or deletions) would also constitute a mutagenic effect. Regardless, three reviewers felt that the existing definition of mutagenicity was too narrow to allow for flexibility as the science advanced and the understanding of mutagenicity and MOAs evolved.

- A reviewer felt that the overly broad discussion of genotoxic endpoints clashed with the document's narrow definition of mutagenicity. This reviewer thought that the Framework, as currently written, did not distinguish between DNA damage endpoints and mutagenic endpoints, but only considered a mutagenic MOA for DNA damaging agents. Another reviewer had this concern initially as well, but felt that Schoeny implied in her presentation that EPA did not intend to narrow the mutagenicity definition so strictly.
- A reviewer noted that agent interactions with proteins could induce mutations; however, the definition of mutagenicity currently presented in the Framework excluded these types of mutations. This reviewer felt that EPA's definition of mutagenicity only considered agents that directly interacted with DNA. Two other reviewers agreed that defining mutagenicity as including only agents that interacted with DNA was inaccurate.
- A reviewer felt that, during real-world application, risk assessors would consider additional data that provided information about the mutagenic MOA for cancer. However, as written, the Framework outlines a process for assessing the mutagenic MOA based on a limited data set.
- A reviewer said that the definition presented in the Framework did not lend itself to assessing a mutagenic MOA with respect to cancer endpoints. Another noted that expanding the definition of mutation to include indirect interactions that resulted in mutations, such as those by agents (*e.g.,* bleomycin, radiation) that generated reactive oxygen species (*e.g.,* hydrogen peroxide, superoxide radicals, and hydroxyl radicals) would be desirable and would consider cancer endpoints.
- The Framework specifically referred to interactions that occurred early in the chain of events leading to a cancer endpoint. One reviewer questioned if use of the term "early" would exclude later events. This reviewer thought that EPA should be careful about using the term "early." Other reviewers agreed that interactions leading to mutations could occur later in the process. They thought that EPA used the term "early" to mean initiating.
- Reviewers also discussed the use of data derived from low-dose assays. A reviewer stated that generating low-dose mutagenicity data was possible using a spontaneous mutation assay. This type of assay would more closely resemble real-world situations than current mammalian cell mutagenicity studies. This reviewer recognized that the panel had not

been charged with requesting or outlining data or assay needs, but wanted to mention the possibility. Scientists could measure both mutation rates and frequencies, and calculate a slope factor for a dose-response curve, based on results from low-dose assays. These assays would not define the tumor type (*e.g.,* liver or lung tumor in humans), but would provide data supporting an increase in tumor rates over background levels. Several reviewers questioned the need for including a reference to low-dose assays in the Framework. One thought that very little data have been generated by standard mutagenicity studies, and even less data have been generated using low, environmentally relevant concentrations. Another questioned the usability of the data in the context of considering MOAs relevant to specific tumors (*e.g.,* liver or lung tumors in humans). A reviewer noted that a limited number of cell types exist for mutagenicity assays, as such, assays for specific tumors are infeasible.

Reviewers provided recommendations during their discussions (although individuals did not necessarily agree with all of them). These recommendations included the following:

- A reviewer suggested presenting a broader definition of mutagenicity in the context of the complexities associated with mutagenicity. A reviewer also suggested that the broader definition include the flexibility to evolve with changing science.
- A reviewer thought that EPA should include tables and examples illustrating the implications of the mutagenicity definition for applying the Framework.
- EPA should include text outlining the different types of mutagenicity that existed and describing how the Framework would apply to each type, recommended another reviewer.
- A reviewer suggested that EPA add a definition of genotoxicity in its broadest context and describe how the term "genotoxicity" would encompass a mutagenic MOA for cancer, which could be illustrated by use of Venn diagrams. Mutagenicity would be considered a subset of genotoxicity. Others disagreed that mutagenicity was a subset of genotoxicity; some aspects of mutagenicity fall beyond the definition of genotoxicity. One felt that they are overlapping sets. All agreed that individual scientists have varying, and sometimes conflicting, definitions of genotoxicity versus mutagenicity and the relationship between these terms.
- Because the current definition was inaccurate in considering only direct interaction with DNA, a reviewer wondered if the panel should consider the different targets that result in mutagenicity. Most instances involved DNA, but other targets also affected mutagenicity.

4. Reviewer Discussion: MOA Framework

- ***Does the document provide a useful framework for determining a mutagenic MOA for carcinogenicity?***
- ***Is the information on the application of the Framework sufficiently clear and complete to be useful?***
- ***Does the Framework provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data generally available for consideration? If not, what additional elements might be useful to include?***
- ***For each step, discuss its application (given the current state of science) and what types of additional information might be added (remembering that this document cannot suggest or require additional testing).***
- ***Does this document achieve the goal of providing a framework for organizing data, determining their relevance, and considering issues in a mutagenic MOA for cancer? If not, what changes could be made to improve the evaluation of MOA information while remaining consistent with the Cancer Guidelines?***
- ***Sections 2.4.9 and 2.4.10: Please comment on the discussion of the use of data in supporting a MOA for carcinogenesis in animals and humans.***
- ***Section 2.2 (Acceptance and Quality Criteria): Is this section transparent to the reader? Does it adequately describe how data quality can be evaluated? If not, how could this section be expanded or improved?***
- ***Section 2.4: Does knowledge of the type of mutational event or the mutational spectra contribute to the WOE for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.***
- ***Aneuploidy: Are the discussions of aneuploidy adequate? If not, how should they be expanded?***
- ***Section 2.4 (last paragraph): Please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.***

During this portion of the meeting, reviewers provided comments and suggestions that addressed their general concerns regarding the Framework. They did not necessarily discuss each charge question individually. Instead, reviewers stressed that their pre-meeting comments and the public comments contained details supporting the general issues raised during their discussions, as well as responses to the specific charge questions. They recommended that EPA carefully review these comments and consider them as they revise the Framework.

In considering the evaluation processes presented in the Framework, the reviewers discussed the following concerns and issues.

- Reviewers felt that EPA should revise the Framework to be a stand-alone document to the extent feasible. They thought that requiring users to repeatedly access other documents (*e.g.,* Cancer Guidelines, Supplemental Guidance) was cumbersome, and would delay progress in conducting risk assessments based on MOAs for mutagenic chemical carcinogens.
- In the written comments, one reviewer suggested a revised document outline that would more clearly present the process for determining a mutagenic MOA. During the meeting, reviewers discussed different options for reorganizing the Framework and referred EPA to the written comments for more detailed information and suggestions.
- Reviewers noted that evaluating the mutagenic MOA was a complex process; the Framework should reflect this complexity. They felt that the discussions and conflicting views expressed during the meeting illustrated this complexity. One reviewer suggested that the Framework only provide a broad description of the evaluation process, and avoid details such as designating specific applications of results from specific assays. This format would provide professional risk assessors with the maximum flexibility to determine whether a specific chemical had a mutagenic MOA and to conduct risk assessments.
- One reviewer noted that a dichotomy existed between how the Framework presented a weight-of-evidence approach for assessing available genotoxicity and mutagenicity data for an agent versus the weight-of-evidence for MOA. The Framework did not link nor integrate these processes. The Framework should walk a risk assessor through the process of conducting the weight-of-evidence analyses and considering the totality of the available data, including illustration by case examples. In addition, this reviewer was uncomfortable with how the Framework presented the weight-of-evidence approach for examining data from screening assays out of context of a mutagenic MOA for cancer. The document did not frame how a risk assessor should proceed in examining data from these assays.
- Another reviewer thought that evaluating a possible mutagenic MOA for cancer endpoints involved a two-step process that considered the questions: 1) Is the agent mutagenic and 2) is the mutation responsible for a cancer endpoint? Another reviewer disagreed. This reviewer felt that the process was more complex and involved the systematic consideration of available data in the context of how mutagenicity contributes to a hypothesized MOA for cancer in animals and its associated human relevance. If evaluations under the Framework supported a risk analysis, than these evaluations must be transparent and systematic. This reviewer thought that the Framework, as currently written, did not sufficiently demonstrate or illustrate the evaluation process.
- A reviewer thought that if a risk assessor applied the Hill criteria in evaluating data for a mutagenic MOA, the minimal data sets typically available would fail to prove data consistency. The Hill criteria may apply to other MOAs, but little data existed to support temporality or dose-response for a mutagenic MOA.
- Reviewers disagreed about the need to consider alternate MOAs beyond a mutagenic MOA. One suggested that the evaluation process included eliminating other MOAs. Another emphasized the need to evaluate the totality of the available data as support for

hypothesized modes of induction of tumors. A third reviewer, however, felt that the Framework focused on mutagenic MOAs, so evaluating other MOAs was unnecessary. A fourth reviewer wondered how a risk assessor who was only focusing on a mutagenic MOA would evaluate an agent that acts under a mutagenic MOA at extremely high doses (*e.g.,* irrelevant to human doses) and an epigenetic MOA at a low dose. The third reviewer responded that a risk assessor would need to evaluate agents with evidence of a mutagenic MOA at any dose.

- Several reviewers noted that many of the assays discussed in the Framework were intended to support hazard identification for an agent. As such, the Framework needed to provide guidance on how data from hazard identification assays could be applied to MOA evaluations.
- One reviewer expressed discomfort about the use of screening assays in evaluating a mutagenic MOA, as presented in the Framework. This comment led to a discussion about the need for professional judgment and agent-by-agent considerations. For example, a reviewer noted that professional judgment was needed in determining an adequate data level for evaluations. Another reviewer questioned how the Framework addressed agents with a mutagenic MOA in concert with high cytotoxicity (*e.g.,* 90% cell death), for example. In real-world situations, the impact on an organism of the cell death would outweigh the impact of mutations. Another reviewer felt that the objective to incorporate data above defaults, as outlined in the Cancer Guidelines, emphasized EPA's move toward the use of more relevant tissue-specific data that better informs weight of evidence for mutagenic MOAs for cancer and subsequent dose-response analysis.
- A reviewer suggested that EPA add case studies to illustrate the processes presented in the Framework. A second reviewer suggested that aflatoxin B1 would be an excellent positive control as an example of a chemical carcinogen with a clearly mutagenic MOA. Aflatoxin B1, which forms DNA adducts and is strongly mutagenic could serve as a case study to calibrate the MOA process for a strong mutagenic carcinogen acting by a mutagenic MOA.
- The Framework did not provide guidance for agents with multiple MOAs, according to two reviewers.
- A reviewer felt that, in many cases, data for evaluating an agent's MOA were limited and might not be available, but that application of the framework in these data poor cases could inform the development of relevant information.

Several reviewers expressed concern that the Framework guided risk assessors to assume that an agent did not have a mutagenic MOA if supporting data were lacking.

- A reviewer felt that the assumption of no mutagenic MOA in the absence of clear evidence shifted the burden of proof away from the agent's producer to EPA. Following this change in default assumptions or responsibilities, a risk assessor would not apply ADAFs for many agents until additional data were collected. Producers of an agent would have no incentive to study an agent's MOA and possibly identify a mutagenic MOA. Conversely, assuming the need to show non-relevance of a mutagenic MOA in the

absence of clear evidence would provide a strong incentive for the proposed user or producer of an agent to fully understand an agent's MOA.

- A reviewer noted that many agents would fall under the assumption that a mutagenic MOA existed because these agents were mutagenic and because data supporting a non-mutagenic MOA were absent.

The reviewers recognized that EPA could not recommend specific assays or data requirements within the Framework, but they discussed the need to provide guidance regarding the types of data and assays that would be most useful for evaluating a mutagenic MOA.

- Two reviewers thought that EPA should clearly identify the assays and data that would be critical in identifying a mutagenic MOA versus the assays that would be desirable in providing further support. They felt that risk assessors could use the Framework to highlight data gaps, especially for agents with a dearth of information. One of these reviewers emphasized that risk assessors could not review assay results as stand-alone information. They must consider the total data set, including cancer bioassays and dose-response data that indicated that the agent was a carcinogen and data that were not necessarily agent-specific (*e.g.,* data regarding compound groups). Throughout the discussion, this reviewer emphasized the need to review the complete data set in the context of a mutagenic MOA for cancer endpoints.
- Assuming that a large number of agents had little or no data regarding tumor formation, but that they did have data from screening assays, one reviewer suggested that EPA add guidance regarding how researchers should proceed in gathering additional relevant data. This guidance, which EPA could illustrate in a diagram, could inform researchers about how to best move forward in examining an agent and highlighting data gaps. An extensive early data set existed for ethylene oxide, for example. This data set, however, was not necessarily useful in assessing a mutagenic MOA for the compound.
- A reviewer felt that the Framework should emphasize assays that identified heritable events in mammalian cells when evaluating a mutagenic MOA for cancer endpoints. Without data from mammalian cells, the data set would be weaker. However, EPA should acknowledge that mutagenicity data for target cells (*e.g.,* thyroid follicular cells) were often unavailable. This reviewer suggested that the Framework discuss the applicability of surrogate tissues (*e.g.,* liver), especially from relevant species versus cell lines or bacteria. This reviewer cautioned, however, that EPA should avoid bogging down the Framework with a lengthy discussion of surrogate endpoints. Another reviewer agreed that assays for specific targets might not exist.
- The dose and the route of exposure affect the results of studies assessing mutagenicity versus carcinogenicity, noted a reviewer. Studies that do not consider these factors are not necessarily comparable, nor do they necessarily provide information about mutagenic MOAs.

In discussing the need for guidance regarding additional data needs, reviewers suggested that EPA create a diagram or figure listing the various assays and studies available to assess genotoxicity and/or mutagenicity.

- One reviewer suggested that this diagram list the various assays and studies in descending order of relevance (*i.e.,* most relevant to least relevant). The diagram could also indicate a minimum level of data required to determine a mutagenic MOA. This designation of a minimum data set would not replace professional judgment, but rather would inform risk assessors about the level of data needed for an evaluation. Most agreed that this type of diagram would be useful. They disagreed about whether the diagram should present assays in descending or ascending order. A reviewer suggested that the diagram move from the least to the most relevant data. From a toxicity perspective, human data might be the most relevant, but from a public health perspective, human data were the least desirable.

  In addition, one reviewer felt that indicating a minimum data requirement was inappropriate. Risk assessors should review the available data based on a continuum that was context dependent. As such, no single point in the diagram could serve as a cut off for determining if data were sufficient or insufficient for evaluating a mutagenic MOA.
- During this discussion, a reviewer reiterated that the Framework should guide risk assessors in evaluating the data set as a whole. The diagram could indicate increasing relevance by moving from simple screening assays to more detailed quantitative dose-response data for specific targets. Knowing this continuum of information could inform risk assessors about data gaps. This reviewer thought that a diagram could illustrate how the data were interrelated and how information moved from screening level data to more detailed dose-response data. Another agreed that the diagram could illustrate the movement from a data-poor to a data-rich situation for an agent.
- The diagram could also provide an exit point from the evaluation process if the data proved or disproved a mutagenic MOA, suggested a reviewer. Two reviewers disagreed and thought that a risk assessor should examine other MOAs if possible; one stated that the Framework should not imply that risk assessors should avoid reviewing available data.
- A reviewer felt that this diagram would provide risk managers with an indication about the level of uncertainty associated with an evaluation. Another noted that regulators were flexible in understanding uncertainty and the need for case-by-case considerations.
- Vinyl chloride and aflatoxin B1 were agents with extensive data sets and would be considered ideal for evaluating a mutagenic MOA. As such, a reviewer suggested that EPA add vinyl chloride and alfatoxin B1 as two case examples to illustrate the data hierarchy presented in this recommended diagram.
- A reviewer also suggested adding a Venn diagram that illustrated the relationship and overlap between genotoxicity and mutagenicity assays.

During the discussions, the reviewers noted the need for case studies to illustrate the concepts and processes described in the Framework. One reviewer thought that these case studies would help risk assessors understand how the data were interrelated and supported a mutagenic MOA. The case studies would also provide a common understanding of the Framework. As illustrated by the discussions among the reviewers during this meeting, the Framework did not clearly

outline the processes associated with evaluating a mutagenic MOA. Others agreed that case studies would improve their understanding of the document and would help risk assessors understand the data needs for conducting an evaluation of a mutagenic MOA. A reviewer also suggested that EPA use these case studies to test the processes outlined in the Framework.

Reviewers did not believe that Section 2.2 ("Evaluate the Data against Current Acceptance and Quality Criteria") was transparent. One reviewer noted that the section included insufficient information to assess if the process for evaluating data was appropriate. This reviewer wondered if the Framework implied that risk assessors should follow the criteria outlined in the International Conference on Harmonization. The process was unclear. Another viewed this section in terms of cytotoxicity data and, in the pre-meeting comments, framed responses to the charge question in light of cytotoxicity. A third indicated that the data acceptance and quality criteria were developed in the context of hazard identification, not a mutagenicity MOA evaluation. This reviewer noted that the Framework must detail the data quality criteria (*e.g.,* GLP conditions, data rigor) if the results of the mutagenic MOA affected regulatory decisions. For example, when reviewing data, FDA required that data met a minimum quality standard and then considered the body of data.

Regardless of what comprises constitutes a minimum data quality standard for a mutagenic MOA evaluation, one reviewer felt that the Framework must present a decisive standard for acceptable data quality. Another reviewer agreed and also suggested that the standard should consider data quality in terms of supporting mutagenicity MOAs for specific tumours versus hazard identification. A reviewer noted that no validated *in vivo* genotoxicity assays exist.

With regard to Sections 2.4.9 and 2.4.10 ("Is the Mutagenic MOA for Carcinogenesis Supported in Animals or *In Vitro*?" and "Is the Mutagenic MOA for Carcinogenesis Supported in Humans?"), reviewers indicated that their written pre-meeting comments presented detailed comments and examples in response to charge questions related to these sections. One suggested expanding these sections to improve the Framework's transparency. For example, these sections could discuss the qualitative and quantitative concerns that arise when extrapolating animal data to human data. Another reviewer agreed that including these concerns was important, especially in terms of the quantitative dose-response data and toxicokinetic data. A third reviewer expressed concern about interpreting genotoxic endpoints in the absence of context about the purpose of these assays. Most assays detected effects resulting from short-term (*i.e.,* 6 to 8 hours) exposures versus chronic (*i.e.,* years) exposures. This reviewer noted that this is a very complex subject.

Another reviewer viewed these sections in terms of interpreting data from studies such as those found in the National Toxicology Program database. Agents usually considered genotoxic acted as carcinogens in multiple animals and at multiple sites. A reviewer noted that generalities were difficult. This statement, for example, would not apply to agents needing metabolic activation. This reviewer was concerned that risk assessors may incorrectly interpret the converse to be true—an agent that is not a multi-animal or multi-site carcinogen did not have a mutagenic MOA. Overall, this reviewer felt that the Framework included many generalities. Another noted that the scientific community clearly accepted that effects seen in multiple tissues during a bioassay were suggestive of a mutagenic MOA. But these results did not prove a mutagenic MOA. Reviewers agreed that exceptions to generalities exist.

5. Reviewer Discussion: Additional Charge Questions

- ***For groups of similar compounds, could a mutagenic MOA be supported solely by structure activity relationships (SAR) or analogy? Apart from groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by SAR or analogy? If not, what additional information might be needed?***
- ***Section 1.2: Is this section clear and sufficiently complete in distinguishing how the use of mutagenicity in the Framework differs from its use by other federal agencies? If not, how can this section be improved and what specific other uses of mutagenicity data for regulatory purposes might be addressed?***
- ***Which version of Figure 1 best captures the steps proposed in the Framework and why?***
- ***Are the appendices useful for determining a mutagenic MOA for carcinogenicity? How can their usefulness and clarity be improved?***

Reviewers disagreed about the use of SAR in supporting a mutagenic MOA. They agreed that SAR were a useful tool, but they disagreed about the application of SAR. During their discussions, they asked EPA to clarify the intent of the charge question related to SAR. Schoeny indicated that this charge question reflected a specific inquiry about whether or not SAR could serve as the sole basis of determining the presence or absence of a mutagenic MOA.

- Two reviewers felt that, if a risk assessor were considering a mutagenic MOA for cancer endpoints, then additional data beyond the SAR would exist. As such, a situation in which only SAR were considered could not exist. One of these reviewers noted that the Framework described a process of using the full body of evidence to evaluate a possible mutagenic MOA. This reviewer assumed that some level of mutagenicity data would be available to assess the adequacy of the SAR. The charge question should not imply that mutagenicity data were unnecessary if SAR were available. The other emphasized that the Framework should note that risk assessors must interpret SAR using expert judgment. This reviewer felt that the data evaluation process was circular, and therefore considering SAR alone was not possible. Interpretation of specific subsets of available data is necessarily informed by other existing data.
- Three other reviewers stated that SAR could not serve as the sole basis for determining that a mutagenic MOA did or did not exist. For example, the characteristics of an agent with an epoxide as a structure element could not be predicted by SAR alone because reactivity among epoxides varied widely. As such, a reviewer advised against assuming that a specific component of an agent drove the agent's MOA.

  For well-studied compound groups, such as polycyclic aromatic hydrocarbons (PAH), SAR could be used to prioritize concerns within the group of PAHs and focus evaluation efforts on the agents most likely to have a mutagenic MOA. These would include PAHs that possessed a "Bay-Region" and could be predicted to be metabolized effectively to

Bay-Region diol-epoxides that were stable enough to bind to DNA and cause mutations. Quantum mechanical calculations could make these predictions, but then mutagenicity tests should be conducted on priority compounds

One of these reviewers also noted that a better charge question regarding SAR would have been, "What could SAR be used for?", and noted that SAR are useful for prioritization, but should not be used alone to predict a mutagenic MOA.

These reviewers noted that EPA's Office of Water used SAR because this office handled thousands of agents. This office, however, used SAR cautiously and required experimental data to support SAR. In some cases, the evidence supporting SAR was strong, but these cases were rare and should still be supported by experimental data. Another reviewer stated that FDA considered SAR to prioritize agents and facilitate decisions. For example, if experimental results were inconclusive, then SAR may be able to help inform results. SAR, however, would not be used alone.

- The sixth reviewer felt that, in the absence of other data, well-established SAR for well-studied groups of compounds could inform regulatory decisions about one agent within the group. From a risk assessment context, this reviewer would be willing to regulate an agent based on SAR if SAR were part of the continuum of data potentially useful for evaluating possible mutagenic MOAs. For example, SAR could provide information about what assays would be most useful for nitrosamines. This reviewer also noted that the Framework did not reference a specific database or data set known to provide reliable information about SAR.

In response to reviewer inquiries, Schoeny noted that EPA wrote the Framework to be as flexible as possible, but still provide guidance. In the past, EPA regulated some agents (*e.g.,* arsenic) based on human data and in the absence of supporting animal models. The current default assumed an unknown MOA unless an MOA was proven. If the MOA was unknown, then risk assessors applied linear extrapolation.

A reviewer agreed that the Framework attempted to provide guidance that was uniformly applicable to any circumstance. Another reviewer felt that the Framework was not fully developed enough to meet this need, which was a problem with the document.

One reviewer expressed concern that if SAR were associated with specific endpoints and served as the basis for a decision regarding a mutagenic MOA, then no incentive existed to conduct additional larger studies to confirm the MOA. This reviewer suggested that EPA define SAR in the Framework. Another agreed that EPA needed to be careful in the use of terminology surrounding SAR and quantitative structure activity relationships ([Q]SAR).

Reviewers agreed that Section 1.2 ("Regulatory Uses of Genetic Toxicology Data") did not clearly and sufficiently distinguish how the use of the term "mutagenicity" in the Framework differed from its use by other agencies. Schoeny indicated that EPA included this section as a consequence of the inter-agency review process and the concern that various agencies used different definitions of mutagenicity. This section attempted to highlight the existence of many definitions. Schoeny also noted that the definition of mutagenicity varied between EPA offices,

programs, and regions. Developing a consistent definition throughout EPA, however, was infeasible at this time.

Reviewers provided the following comments and suggestions regarding Section 1.2 of the framework.

- The section seemed misplaced and detracted from the flow of the Framework, thought one reviewer. This reviewer suggested moving the text to an appendix and including examples of how various agencies use genotoxicity data. Another agreed that the text detracted from the discussion of MOA.
- Several reviewers encouraged EPA to develop a consistent definition of mutagenicity and genotoxicity across the agency. One thought that these definitions should be based on the conventional terminology that most genetic toxicologists and molecular carcinogenesis researchers currently employ.
- One reviewer thought that clarifying the definition of mutagenicity, as suggested during the panel discussion, would improve this section. Another suggested that EPA consider the article prepared by Cimino (2006), which was referenced in the Framework. This article reviewed how EPA and other agencies generated and used various data, which highlighted the differences in how EPA and other agencies defined mutagenicity and other terminology. This reviewer also thought that the Framework should highlight the implications of the varying definitions and uses of the terminology by different agencies. A reviewer suggested that EPA cite these articles as supporting documentation to the Framework. Schoeny noted that the Framework cited these articles as extensively as possible.

Reviewers noted that various agencies used the results from genotoxicity and mutagenicity assays for different purposes. The Framework discussed the use of these assays to support evaluation of potential mutagenic MOAs. Reviewers provided the following comments regarding the Framework's presentation of genotoxicity data and its uses.

- One reviewer objected to the concept that genotoxicity data alone could support an evaluation of possible mutagenic MOAs for cancer. This reviewer noted that tumor data identifying an agent as a carcinogen would exist and should also be considered. EPA needed to improve the text describing how to apply genotoxicity data to evaluating an MOA. Two other reviewers agreed that the Framework was insufficient in discussing the implications of using genotoxicity data to support MOA versus hazard identification.
- A reviewer noted that the article by Dearfield (2005), as cited in the Framework, listed possible uses of genotoxicity data. Supporting MOA evaluations was the third application. This reviewer felt that a discussion of other agencies was less important than clearly outlining the uses and limitations of genotoxicity data in supporting MOA.
- To this end, a reviewer suggested that EPA add text highlighting that some assays were more useful in evaluating MOA than others. Another suggested that the text also discuss that assays were developed for different purposes, and that some assays were not relevant for evaluating mutagenic MOA for specific tumors.

- A reviewer thought that, when assessing a mutagenic MOA, risk assessors would need to ask: 1) Was the agent a carcinogen? 2) Was the agent a mutagen based on results from assays? and 3) Did the agent act using a mutagenic MOA? The Framework, as written, did not clearly articulate this process. Reviewers considered if a risk assessor answering "no" to either of the first two questions should proceed with the process outlined in the Framework. In the context of the Framework, two thought that a risk assessor should not continue the evaluation if a decision point indicated that a mutagenic MOA was absent. One of these, however, noted that a risk assessor may want to extend analyses to other MOAs. Another thought that a risk assessor should carry an evaluation to its conclusion even if evidence indicated that a mutagenic MOA was absent. Regardless of the results, the evaluation would provide useful information.

One reviewer noted that the overall level of discussion and discord among reviewers indicated that the Framework was unclear in the definition of mutagenicity and MOA.

Reviewers suggested that EPA create a new version of Figure 1. Although some reviewers preferred version 1 and others preferred version 2, all felt that neither version 1 nor 2 clearly illustrated the circular process of evaluating a possible mutagenic MOA. One reviewer thought that Figure 1 was misplaced in the Framework and belonged in the discussion of examining the MOA versus the discussion of hazard identification. Version 2 consisted of a figure and an unrelated table. One reviewer felt that a new figure should outline the principal steps in the evaluation process—gathering all relevant data, reviewing and understanding the data in the context of MOA, walking through the Framework, and finally following a feedback loop to continue evaluating and re-evaluating the data. Another noted that key steps in the evaluation process were missing. In revising the figure, one agreed with the public comment suggesting that EPA add references to relevant sections of the text for the various steps presented in the figure.

Two reviewers suggested that case studies would inform the figure format. A case study would illustrate the process of following steps outlined in a figure. Vinyl chloride or aflatoxin B1 could serve as examples of robust data sets. Through presentation of a robust data set, these examples would help risk assessors identify data gaps that may exist for other agents and also calibrate the process for determining that a carcinogen had a mutagenic MOA. Another review stated that case examples would also illustrate how various data (*e.g.,* results from genotoxicity studies) would be used in evaluating a mutagenic MOA.

Reviewers felt that some of the appendices were more useful than others. One felt that the appendices were not helpful in the context of MOA. Suggestions for improving the appendices included the following.

- An appendix should outline the data needed for an evaluation, and especially detail how these data informed dose-response.
- The literature organization table was limited to standard testing assays, and different types of studies other than those used for hazard identification may be needed for a MOA evaluation. A reviewer thought that the rich database of available information and materials should be mentioned.

- EPA could present the appendices in terms of key events, which drove the process for moving through evaluation of a mutagenic MOA and informing dose-response.

6. Reviewer Discussion: Additional Comments and Concerns

- ***Is the document clear, complete and objective?***
- ***Are major limitations and assumptions clearly defined?***
- ***Does the panel have any other comments or concerns (both strengths and weaknesses) that have not already been discussed?***

The panel agreed that they had addressed these first two questions during their discussions under the other topic areas. They also stressed that their pre-meeting comments and the public comments provided important details that expanded on the comments and concerns discussed during the meeting. The panel reiterated the need for EPA to fully review and consider both the public comments and the written pre-meeting comments submitted by the panel.

The reviewers also provided the following additional comments.

- Several reviewers felt that substantial revisions to the Framework were necessary to address the concerns voiced during this meeting and in written comments. They suggested and strongly supported the idea, that EPA conducts a second peer review after these revisions.
- Recognizing that limitations may exist in the extent of revisions that are feasible, a reviewer suggested that EPA improve discussions regarding the data types that best informed, or conversely did not support, a conclusion regarding a mutagenic MOA.
- The Framework did not discuss mutations as a second or third event in process of an agent resulting in a cancer endpoint. For example, would EPA consider agents that caused epigenetic mutations that then affect DNA mutations as a mutagen under the Framework? This reviewer said that the Framework did not discuss the sequence of events as they relate to a determination regarding a mutagenic MOA. Another reviewer thought that the Cancer Guidelines would identify an epigenetic MOA in these instances.
- Reviewers varied in their opinions about possible uses of dose-response data and provided a number of concerns about the use of dose-response data in the Framework. Most, however, agreed that the Framework lacked in its discussion of how data on the mutagenic MOA informs subsequent dose-response analyses.
    - The availability of tumor data would lead a risk assessor to consider a possible mutagenic MOA for an agent. The data on rate limiting key events in the target tissue would inform subsequent dose-response analyses.
    - Mutations occurred for many reasons, not just agent exposure. As such, mutations are not purely related to the dose-response curve.
    - One reviewer noted a preference for data that provided comparable dose-response curves for both mutations and tumors. This reviewer noted that such data exist for in vitro studies of mammalian cells where mutagenesis and morphological/ neoplastic cell transformation for mutagenic carcinogens are often dose-dependent over the same dose ranges. Another reviewer, however, noted that

many concerns existed about comparing these dose-response curves because they were not the same.
    - The dose-response data for mutations must be biologically relevant. For example, the usability of dose-response data for mutations at 90% cell death was limited. The mutagenicity data must be considered in context with toxicity data as well as dose to the tissue.

- A reviewer again suggested adding vinyl chloride or aflatoxin B1 as case studies because these were data-rich agents that clearly acted by a mutagenic MOA for carcinogenesis. Another reviewer agreed that these agents would illustrate how to conduct an analysis of the mutagenic MOA, but this reviewer was unsure if these examples would provide the appropriate range of possibilities.
- One reviewer noted that the designation "unable to determine" would apply to many agents.

Appendix A

List of Peer Reviewers

Peer Review Meeting: EPA's Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity

Navy League Building
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April 4, 2008

Reviewer List

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Appendix B

Charge to Peer Reviewers

Issues for External Peer Review – U.S. EPA *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity (Framework)*

1. The purpose of the *Framework* is to provide an overall process by which chemicals can be evaluated and a determination made regarding whether the chemical has a mutagenic mode of action (MOA) for carcinogenicity. MOA is described as the key decision in risk assessment in U.S. EPA's 2005 *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*, and the accompanying *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (Supplemental Guidance)* highlights the importance of ascertaining whether a chemical has a mutagenic MOA. The *Framework* document recognizes both the on-going research in this area, as well as the potential limitations of available data for making this determination.
   a. Please comment on whether the purpose for the document is clear.
   b. Does the document provide a useful framework for this determination?
   c. Is the document clear, complete and objective?
   d. Are major limitations and assumptions clearly defined?

2. Given the inconsistency in the scientific community of genetic toxicologists regarding the definition of mutagenicity, the *Framework* (section 1.4) proposes an *operational* definition of "mutagenicity" for a very limited use, i.e., determination of a mutagenic MOA *for carcinogenicity* under the U.S. EPA *Cancer Guidelines* and *Supplemental Guidance*. Please discuss whether this definition is useful and appropriate, considering the limited application in this *Framework*.

3. Section 1.2 (uses of mutagenicity data) was added in coordination with and at the request of other Federal agencies that wished to distinguish their use of such data from the use in this *Framework*.
   a. Is this section clear and sufficiently complete to accomplish that goal? Please suggest recommendations to improve this section.
   b. If this section is not complete, what specific other uses of mutagenicity data for regulatory purposes might be addressed?

4. Which version of Figure 1 best captures the steps proposed in the *Framework*? Please identify the basis for your recommendation.

5. The MOA framework is defined in the *Cancer Guidelines.* The steps are repeated in the *Framework* with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.
   a. Is the information in this *Framework* on the application of the *Cancer Guidelines* MOA framework sufficiently clear and complete to be useful?
   b. Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data that are generally available for consideration? If not, what additional elements might be useful to include in the *Framework*?
   c. Please review each step, e.g., "key events," "dose-response relationships," "temporality," and discuss what types of additional information might be added

(remembering that this document can not suggest or require additional testing). Given the current state of science, please comment on the application of each step in MOA framework.

6. Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader? Does it adequately describe how the quality of the data can be evaluated? If not, how could it be expanded or improved? Please provide specific recommendations.

7. Are the discussions of aneuploidy adequate? If not, how should they be expanded? Please provide specific examples and detailed information regarding recommendations.

8. Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutational event, or knowledge of mutational spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.

9. Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available .

10. The WOE for determining an MOA for carcinogenicity is described and determined in the U.S. EPA *Cancer Guidelines*. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss if we have achieved this goal. If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?

11. For groups of similar compounds, e.g., nitrosoamines or polycyclic aromatic hydrocarbons (PAHs), could a mutagenic MOA be supported solely by structure activity relationships or analogy? Apart from the consideration groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by structure activity relationships or analogy? If not, what additional information would you think might be needed?

12. Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (Section 2.4.9) and in humans (Section 2.4.10).

13. Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

Appendix C

Reviewer Pre-Meeting Comments

Notice

Pre-meeting comments were prepared by each consultant individually prior to the meeting. They are preliminary comments only, and are used to help consultants become familiar with the document and charge questions, develop the agenda, and identify key issues for discussion. During the meeting, consultants may expand on or change opinions expressed in their pre-meeting remarks and may introduce additional issues. For these reasons, pre-meeting comments should be regarded as preliminary and do not reflect the final conclusions and recommendations of individual consultants. These pre-meeting comments will be included as an appendix in the meeting summary report, along with other background materials.

Elaine Faustman

TECHNICAL CHARGE TO PEER REVIEWERS

External Peer Review of U.S. EPA's draft *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity (Framework)*

Task Order No. 9
Contract No. EP-C-07-024
February 20, 2008

PRE-MEETING COMMENTS ARE DUE TO ERG BY CLOSE OF BUSINESS, MONDAY, MARCH 24, 2008

CHARGE QUESTIONS

1. **The purpose of the *Framework* is to provide an overall process by which chemicals can be evaluated and a determination made regarding whether the chemical has a mutagenic mode of action (MOA) for carcinogenicity. MOA is described as the key decision in risk assessment in U.S. EPA's 2005 *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*, and the accompanying *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (Supplemental Guidance)* highlights the importance of ascertaining whether a chemical has a mutagenic MOA. The *Framework* document recognizes both the on-going research in this area, as well as the potential limitations of available data for making this determination.**
    - **a. Please comment on whether the purpose for the document is clear.**
    - **b. Does the document provide a useful framework for this determination?**
    - **c. Is the document clear, complete and objective?**
    - **d. Are major limitations and assumptions clearly defined?**

1a. The development of framework for determining modes of action (MOA) for carcinogenicity is a laudable but challenging goal. In reviewing the materials provided I have the following initial comments:

I feel that the goal of the document to ensure that "nothing in this document (framework) should be interpreted as superseding either the Cancer Guidelines or the Supplemental Guidance" is laudable however I think the framework fails to uphold this goal.

b. The document repeatedly states that there are multiple uses of genetic toxicology assays and data by regulatory agencies (Page 6, lines 21-22) and that genetic toxicologists have multiple definitions of "mutagenic" and "genotoxic" (Page 8, lines 19-28) and that EPA has differing definitions of "mutagenicity" depending upon context (Page 8, lines 30). Given this extremely complex context for any discussion of MOA for carcinogenicity and mutagenicity, EPA should require any proposed framework to provide methodical and in-depth clarification and transparency to the discussion. Rather than trying to be brief and not repetitive with existing documents the discussion here is too minimal and abbreviated. The

approaches should be comprehensive and need to clarify the focused definitions used within this proposed framework document within this broader context. The reader is left confused and unclear how to proceed with the framework. The reviewer asks, “If everything is left out of the definition of mutagenicity, is this really a framework for mutagenicity as a MOA for carcinogens?” This does not bring clarity to the proposed user of such a framework and in fact casts serious doubt on the validity of such an approach without tackling the albeit more difficult challenge, but necessary goal of understanding mode of action for mutagenicity in the full definition of mutagenicity used within the agency.

c. Of particular concern to this reviewer is a reversal of previous frameworks and constructs for evaluating mutagenicity. This is present in small aspects such as is seen in the reversal of considerations in WOE in Figure 1v2 page 2, compared to recent proposal and related peer reviewed frameworks for DNA reactive carcinogens (Preston and Williams, 2005).

But most importantly it is present in the very construct on how the weight of evidence is developed. For example, the burden of proof is now to prove mutagenicity as a MOA not prove it is not relevant. This reverses the previous frameworks and importantly violates one of the initial stated goals of the document that guidance in the two EPA reference documents will not be superseded. Of greatest public health concern is the switch in the underlying default assumption for the MOA framework. The proposed approach in the new framework would be to prove mutagenicity as a MOA rather than to displace its relevancy and this is not consistent with the approaches in either the current EPA guideline document for Carcinogen Risk Assessment (EPA, 2005) nor supplemental guidance (EPA, 2005b) nor is it consistent with the International Agency on Carcinogens (IARC) Monographs or ICH testing paradigms. To require proof of mutagenicity shifts the burden of proof and incentives for data generation away from obtaining new scientific information.

The document draws heavily on many references developed by the Technical panel yet in many cases does not reconcile these approaches with other references by agency scientists Cimino, 2006 and Preston and Williams, 2005 nor outside approaches also available in the peer reviewed literature (IARC; Meek et al 2003;or Butterworth, 2006). This is one example where a more complete discussion would be essential. See also comments regarding section 1.4 for completeness.

d. No, assumptions and limitations are not clearly defined. A clear table of assumptions would be needed and explicit information on genotoxicity events not included in the frameworks revised definition for mutagenicity should be discussed. Explain how other mutagenic modes not included in this limited definition would be needed.

It was disconcerting to this reviewer to find many inconsistencies in stated goals within the text.

2. Given the inconsistency in the scientific community of genetic toxicologists regarding the definition of mutagenicity, the *Framework* (section 1.4) proposes an *operational* definition of "mutagenicity" for a very limited use, i.e., determination of a mutagenic MOA *for carcinogenicity* under the U.S. EPA *Cancer Guidelines* and *Supplemental Guidance*. Please discuss whether this definition is useful and appropriate, considering the limited application in this *Framework*.

The 46 lines of text added as section 1.4 in the framework provides little to no clarity or context for an operational definition of mutagenicity. For example, on page 8 lines 19-28, it is interesting to note that although this paragraph states that there are numerous definitions for mutagenicity it does not contain a single reference to support this statement. Add references and more specific comparisons on how these definitions vary.

This reviewer noted that in the detailed review by Michael Cimino (Cimino 2006) of genetic toxicity testing guidelines within EPA, he identifies numerous offices and regulatory contexts that use genotoxicity testing including Toxics, Pesticides, Office of Air Radiation (OAR), Office of Solid Waste and Emergency Response (OSWER), Office of Research and Development (ORD) and Office of Water (OW). This has resulted in 18 OPPTS guidelines in genetic toxicology that are harmonized between the toxics and pesticides offices and which are linked to OCED guidelines. It would seem essential to this reviewer that the definitions used for mutagenicity and outcomes for such tests and across such contexts be evaluated methodically and compared and contrasted with the definition proposed in this document for mutagenicity. These comparisons could then be placed into context with definitions for mutagenicity used and applied in other US regulatory agencies such as the US Food and Drug Administration (USFDA), Consumer Products Safety Commission (CPSC) and Occupational Safety and Health Administration (OSHA). Finally, context for EPA's new definition for mutagenicity should be discussed in the context with some international definitions. This could be accomplished using tables and figures extending those available in the Cimino 2006 reference.

A larger issue for answering questions is a public health question. In essentially all previous contexts for USEPA the burden of providing safety and safe use for a new chemical has been on the proposed developer of the chemical and the default position is that a mutagenic mode of action could exist if positive assay data was shown to be evident until proven not to be relevant for the case. This is also consistent with the MOA approaches proposed in a paper entitled "DNA Reactive Carcinogens: Mode of Action and Human Cancer Hazard" by Preston and Williams, 2005). However, this MOA approach appears to "get lost" in the current document. Definitions used for DNA reactive chemicals seem much

clearer in this short paper than in the document under review. Key events diagrams are also logical (See Table 1, Preston and Williams, 2005) versus the "reversed" version presented in Figure 1 version 2 page 2 (report page 13). However, this reviewer would caution that given the complexity of biological responses under mutatgenicity or genotoxicity that great attention to details is needed in the framework document.

3. **Section 1.2 (uses of mutagenicity data) was added in coordination with and at the request of other Federal agencies that wished to distinguish their use of such data from the use in this *Framework*.**
    a. **Is this section clear and sufficiently complete to accomplish that goal? Please suggest recommendations to improve this section.**
    b. **If this section is not complete, what other specific uses of mutagenicity data for regulatory purposes might be addressed?**

Please see my comments for question 2. The page of text added as Section 1.2 is not sufficient to distinguish uses of data within various federal agencies versus that proposed in this new framework. In my response to question 2, I provide some specifics on how such a discussion should be organized and some discussion on details needed to understand the differences.

4. **Which version of Figure 1 best captures the steps proposed in the *Framework*? Please identify the basis for your recommendation.**

See my concerns about the overall framework context. Because of these concerns it is difficult to choose a specific version. In general, version Figure 1 v1 is better. Why not use or build upon the framework presented by Preston and Williams for DNA reactive carcinogens that builds on the Human Relevancy Framework?

5. **The MOA framework is defined in the *Cancer Guidelines.* The steps are repeated in the *Framework* with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.**
    a. **Is the information in this *Framework* on the application of the *Cancer Guidelines* MOA framework sufficiently clear and complete to be useful?**
    b. **Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data that are generally available for consideration? If not, what additional elements might be useful to include in the *Framework*?**
    c. **Please review each step, e.g., "key events," "dose-response relationships," "temporality," and discuss what types of additional information might be added (remembering that this document can not suggest or require additional testing). Given the current state of science, please comment on the application of each step in the MOA framework.**

a-c: Please see comments above about faults in context for framework, problems in scope of definition and order of considerations. This reviewer did not find useful information in Section 2.4 of the document. In fact in several cases felt numerous examples of oversimplifications are present in Section 2.4. For example on page 21, the framework document states that "consistency with the same effect across different assays supports the WOE for that specific mutagenic effect." What assays? I would not necessarily believe more in a proposed MOA if Ames assay results for strains for strains detecting both point and frameshift mutations were positive for the same alkylating agent. This simplification is too simple, is misleading and confusing. This is just one example of the problem throughout the current framework.

In Section 2.4.1 Key Events pages 23, lines 32-36 and page 24, lines 4-6 the text discusses examples of properties for mutagenicity as the key event. None of these statements is referenced and consistency with peer reviewed literature discussing the complexity of "early" versus "late events" (See Hanahan and Weinberg, 2000) is needed. The Hanahan and Weinberg review states "Further, mutations in certain onco-genes and tumor suppressor genes can occur early in some tumor progression pathways and late in others." (Page 67). The current emphasis in the framework document on early versus late mutational events seems to be very simplistic and misleading. This is an other example of the problematic simplification in the framework document.

6. Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader? Does it adequately describe how the quality of the data can be evaluated? If not, how could it be expanded or improved? Please provide specific recommendations.

The 16 lines in Section 2.2 seem to be very general. Use of examples to illustrate the criteria for judging acceptability should be described in more detail rather than just list a series of references, especially since some of the references i.e. ICH 1995 and 1997 and Cimino, 2006 listed have different definitions and defaults for interpreting mutagenicity data than is proposed by this framework document.

7. Are the discussions of aneuploidy adequate? If not, how should they be expanded? Please provide specific examples and detailed information regarding recommendations.

Please refer to the comments by D. Albertini, as his long and detailed explanation of issues with interpretation of various mutational and chromosomal assays provides an excellent referenced resource. Examples from his information should be included and his excerpts from unpublished IRAC documents on Ethylene oxide would provide good examples and would challenge the framework as proposed.

8. 8. Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutational event, or knowledge of mutational spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.

Many scientific, peer-reviewed papers exist that discuss the complexity of MOA for carcinogenicity yet Section 2.4 applying the MOA framework appears to make this discussion very simple, too simple. Three example points will be discussed.

As mentioned above, Hanahan and Weinberg, 2000 provide an excellent insight into the complexity of events and differential sequence of events possible in cancer generation. The framework should be flexible enough to include these variations and in fact simplistic statement such as "is mutation an early key event in this chemicals induction of cancer?" or "For a chemical to act by a mutagenic MOA, either the chemical or its direct metabolite is the agent inducing mutations that initiate cancer" (Page 23, lines 26-30) are shown to be too narrow for use. See the Hanahan and Weinberg for examples of this significant complexity needed for understanding cancer MOAs. They specifically reject this simplification.

Section 2.4.4 Dose-response relationships (page 25, lines 39 – page 26, lines 1-17)

This brief section on dose-response relationships needs lots of additional considerations. There are no discussions on how extrapolation of acute or short term exposures from in vitro or in vivo assays are related to two year chronically exposed tumor bioassay information. Simple statements that the "key issue is whether the observed dose-response relationships of the initial mutagenic events correspond with the dose-response relationships for tumors." (Page 25, lines 39-40) fail to address such complex dose response issues. Simple, unexplained or illustrated statements such as "An analysis of the absorption, distribution, metabolism and excretion (ADME) aspects of a chemical exposure" (Pg 19, lines 30-34) need details and illustration for the MOA approaches proposed. There are robust and interesting detailed data available for carcinogenicity but this richness of the literature and detailed investigation of the quantitative aspects of MOA are not discussed or illustrated here.

Section 2.4.7 (Pg 27, lines 14-20). The framework documents state that MOAs can be analyzed in conjunction. Please explain and illustrate how this is done.

9. Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.

Page 22 lines 21-28 appear to address the issue of a minimal database by saying that a case-by-case decision can be made to proceed to a MOA analysis. It lists three example situations that would allow the investigator to proceed however how is not given in this section. This would seem to be an important omission as the majority of cases will probably be data poor situations.

10. The WOE for determining an MOA for carcinogenicity is described and determined in the U.S. EPA *Cancer Guidelines*. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss if we have achieved this goal. If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?

See numerous examples listed above that highlight that the proposed goals for the framework have not been met. See detailed comments suggesting that entire framework needs to be examined due to changes in basic premise.
If this framework is to be modified then use established examples in the literature of MOA frameworks for carcinogenicity such as illustrated and discussed in Preston and Williams, 2005 or Meek et al 2005.

Provide at least four examples, case studies that illustrate for the potential user of the mutagenic MOA framework how such a framework would be applied. This is essential for any comprehension or understanding or test of the framework. Such examples should include both data rich and data poor examples.

Since many of the framework approaches will have major implications for assessing susceptibility from early-life exposure to carcinogens (despite repeated assurances that no superseding of the supplemental guidelines is intended) at least two of the case studies should be with chemicals of interest for age specific exposure. One should include urethane due to its complexity of proposed MOA and evidence for age specific effects. Releasing this document with out such tests would not be appropriate.

Again this reviewer feels very concerned that this approach has not been fully evaluated and changing the basic underlying premise for evaluation of the mutagenic MOA is not justified nor tested nor clear

in its potential application nor in its alteration of how public health will be protected. This is especially true as the document starts off with the statements that the various EPA agencies and other agencies use different definitions for mutagenicity and no impact assessment is provided that illustrates how such a narrow proposed definition for mutagenicity will provide clarity and not confusion to this already complex situation.

11. For groups of similar compounds, e.g., nitrosoamines or polycyclic aromatic hydrocarbons (PAHs), could a mutagenic MOA be supported solely by structure activity relationships or analogy? Apart from the consideration of groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by structure activity relationships or analogy? If not, what additional information would you think might be needed?

The SAR relationships for some classes of carcinogens (like those listed above for nitrosamines and polycyclic aromatic hydrocarbons) are robust and this reviewer could envision that our understanding of dialkyl nitrosamines and even cyclical nitrosamines could alone support a mutagenic mode of action. However, the question is not that simple. What consistent, validated SAR databases will EPA accept in their reviews? Does their own Tox Cast program provide a significant resource for answering these questions? Has it been validated? Why was this not listed as an example in the report? Please add other EPA specific and cross program information and examples. Increase literature review and cite here to support such possible approaches. Add information about QA/QC for databases that would provide the basis for such decisions.

12. Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (Section 2.4.9) and in humans (Section 2.4.10).

The two pages devoted to these important concepts are largely without reference, without specificity in examples and difficult to comment upon as it is unclear exactly how this data will be used. For example in section 2.4.9 there is reference made to the Comet assay however this is confusing as is discussed by D. Albertini, this is not a measure of mutation but of several types of DNA damage. In addition numerous forms of the assay exist but the framework document does not provide details to understand what specific forms or if all are being mentioned.

Another example of issues is in section 2.4.10 where reference is made to mutations in cancer-relevant genes in humans however these findings are stated as evidence of "not sufficient evidence" yet no reference is given to support or qualify this statement.

Again, in general a very complex topic is reduced to non-referenced, uneven and brief discussion without examples. Examples of published papers on carcinogenicity MOA illustrate that directed, focused discussions and referenced reviews are possible but these have not been illustrated in the current framework document.

13. Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.

Utility of Appendices

Appendix A: Reverse order of examples starting with in vitro analysis as the data at the bottom of this list would be available first and also could be most important in early discussions about subsequent tests.

Appendix B: This appendix provides a good but much abbreviated review of different mutagenicity testing schemes in use at EPA. The cited Cimino, 2006 provides an even better more detailed and clear discussion of these differences so additional tables from that reference should be considered. However of greater importance is that although these batteries are discussed in this appendix the consistency of how these and various other users of these data differ from the proposed default in this framework is significant in the absence of this difference being discussed in this section. All of the other users explicated or implicitly affirm the concept of proving non relevance of mutagenicity data and to be silent on this consistent difference seems to be ignoring the "elephant in the room".

Appendix C: Examples of the Use of Structure-Activity Relationships in Assessing Mutagenicity
This section is needed however only two references are given and one reference (Deerfield et al 1991) was more general not giving specific information. No mention is made of EPA projects such as Tox Cast or others from the Computational Toxicology program in RTP. Also no mention is made about the large National Cancer Institute (NCI) database on SAR information available from the NCI for evaluating structures and activities of mutagenic chemotherapeutic agents.

Appendix D:

Toxicogenomics: Page 49, lines 19 – page 50, lines 1-15.
The reviewer was interested in the mention of gene expression profiling techniques and results for identifying MOA (J. van Deft et al 2004 and H.E. Ellinger-Ziegelbauer et al 2004). This reviewer would exercise caution in discussing these approaches for discriminating genotoxic versus non-genotoxic carcinogens. Note that van Deft et al 2004 specifically cautions that among the 20 members of compounds evaluated, several notable exceptions (of particular interest to EPA) exist. For example, Trichloroethylene (TCE) was predicted to be genotoxic versus IARC and NTP evaluations. In addition, all of the methylating agents (N=4) evaluated in this study caused minimal changes in gene profiles compared to other recognized carcinogens such as polycyclic aromatic hydrocarbons. Although authors speculate on reasons why these differences exist this seems to highlight the need for additional studies with these new tests prior to use in informing public health.

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

1. Page 6, lines 1-8. These lines state that "The analysis in this framework expands and clarifies discussions found in the Cancer Guidelines and Supplemental Guidance." In this reviewer's opinion only does this document not provide clarity but it adds to confusion by re-defining mutagenicity but it also appears to include a complete policy reversal in how default approaches are handled for mutagenic MOA that challenge basic concepts discussed in the Cancer Guidelines and Supplemental Guidance.
2. Page 6, lines 7-8(also lines repeated at Pg 6, lines 7-10). These lines specifically state that "Nothing in this document should be interpreted as superseding either the Cancer Guidelines or the Supplemental Guidance." However it is silent on what is expected for the EPA 1986 Guidelines for Mutagenicity Risk Assessment. Please specify what was done for this guidance. This is important as the framework report notes that "while heritable mutation is a different adverse health outcome than cancer, both health outcomes involve mutation as part of their etiology" (Pg 5, lines 35-39).
3. Page 6, section 1.2.Regulatory uses of genetic toxicology data. If reviewers are knowledgeable about genetic testing then even these assessments are usually done with some review of dose-response. Isn't this hazard characterization?
4. Page 6, section 1.2. Regulatory uses of genetic toxicity data. This section seems very limited. There are lots of uses – could you site other examples? What about the multiple uses

reviewed and discussed methodically in Comino, 2006? See also my comments on expanding discussion in Appendix B.

5. Page 7, lines 12-17. The document discusses hazard identification and MOA judgments and it says that the framework only focuses on the second of these steps. It also says that it only focuses on carcinogenicity. What does this mean that it ignores heritable germ cell effects? Need to review and clarify language and context.
6. Page 8, lines 1-7. This section discusses mutagenicity as an obligatory early event in carcinogenicity. More details and appropriate references are needed here as this sentence is too simple. See D. Albertini's comments on this framework and Hanahan and Weinberg, 2000 for a more clear discussion of how mutagenicity does not have to be an early event.
7. Page 8, lines 13-15. The statements "It should also be noted that there is no "default MOA" need clarification as does the following statement "The Cancer Guidelines offer some default procedures to use when no MOA can be determined". These statements appear without context yet would appear to be extremely significant. Don't these two sentences differ from the cancer guidelines and supplemental guidance for assessing susceptibilities for early-life exposure to carcinogens? This reviewer needs clarity on this issue.
8. Page 10. Figures/process seems to be reversed and is especially problematic for data poor compounds as presented.
9. Page 11, Figure 1. Shift default when it is unknown whether the chemical has mutagenic activity to considering a mutagenic MOA until proven not to be relevant.
10. Page 13, Figure 1 page 2.Reverse the order of WOE factors to Increasing order of specificity and in detail of availability data.
11. Page 31. Good strong statement about what to do when WOE supports a mutagenic MOA for carcinogenicity. This section 3.0 is consistent with earlier statement that original guidelines are not to be superseded.
12. Page 32, Section 4 Glossary and Acronyms. Please add definition for mutagenicity. Please re-look at the definition of gene mutations. What is a "small-scale" change? Are frameshift mutations considered "small-scale" changes? How do small scale changes differ from point mutations?
13. Page 43, Figure B-1. Figure legend should specify difference between ----- dashed lines and solid lines. What is the "O" 2 lines below specific Locus text?

Final Comments: In reviewing this document this reviewer is reminded of discussions about limiting definitions of other serious endpoints. For example, in developmental toxicity there were suggestions to limit discussions of teratogens to only agents causing birth defects and agents causing embryo or fetal

lethality could not be teratogens as these developing offspring would not make survive to birth. In that discussion EPA provided much needed clarity of the discussion and took a public health protective approach that included all four manifestations of adverse impacts (death, malformation, growth and functional impacts) as significant and as evidence of developmental toxicity. It would be important that EPA could again take the lead in clarifying the risk assessment literature and application with a more comprehensive and integrated definition for mutagenicity.

References Cited

1. Cimino, MC. (2006) Comparative overview of current international strategies and guidelines genetic toxicology testing for regulatory purposes. Environ. Mol. Mutagen, 47: 362-390.

2. U.S. Environmental Protection Agency (U.S. EPA). (2005a) Guidelines for carcinogen risk assessment, EPA/630/P-03/001F, March 2005.

3. U.S. Environmental Protection Agency (U.S. EPA). (2005b) Supplemental guidance for assessing cancer susceptibility from early-life exposure to carcinogens, EPA/630/R-03/003F, March 2005.

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5. Matthews, EJ; Kruhlak, NL; Cimino, MC; Benz, RD; Contrera, JF. (2006) An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: II. Identification of genotoxicants and reprotoxicants using in silico methods. Regul Toxicol Pharmacol 44:97–110.

6. Preston, RJ and Williams, GM. (2005) DNA-reactive carcinogens: mode of action and human cancer hazard. Crit Rev Toxicol 35:673-83.

7. Meek, M. E. et al (2003) A framework for human relevance analysis of information on carcionogenic modes of action. Crit. Rev. Toxicol. 33: 591-654.

8. Butterworth, B. (2006) A classification framework and practical guidance for establishing a mode of action for chemical carcinogens. Regulatory Tox and Pharm 45:9-23.

Robert Heflich

Review of mutagenic MOA Framework. The document takes the framework for determining a MOA that is contained in the Cancer Guidelines and provides additional information on the specific case of determining a mutagenic MOA. Although sufficient background is provided in the Framework to make it reasonably clear by itself, readers are cautioned to refer back to other documents, including the Guidelines. Overall, the basic process of determining a MOA seems straightforward but the Framework stuck me as uneven in its presentation (detail provided for some things and not others; some information of marginal significance; nonideal placement for pieces of information). More importantly, I am left wondering how well the process of determining a MOA espoused by the Framework will work with the datasets that are likely to be available.

The charge questions are given below in bold. My comments follow each question in plain font.

1. **The purpose of the *Framework* is to provide an overall process by which chemicals can be evaluated and a determination made regarding whether the chemical has a mutagenic mode of action (MOA) for carcinogenicity. MOA is described as the key decision in risk assessment in U.S. EPA's 2005 *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*, and the accompanying *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (Supplemental Guidance)* highlights the importance of ascertaining whether a chemical has a mutagenic MOA. The *Framework* document recognizes both the on-going research in this area, as well as the potential limitations of available data for making this determination.**
   a. Please comment on whether the purpose for the document is clear.

The purpose is explained clearly starting with the Preference. I wondered about the difference between a Guideline, Guidance, and Framework. P. 5 stays that the Cancer Guidelines contain a framework and this document is derived from the Guidelines, so I expect that a Framework is less encompassing/more specific than a Guideline. But in other contexts it could be the other way around. These terms seemed to be chosen deliberately, so I expect there is a guideline/framework/guidance for naming these types of documents somewhere.

b. Does the document provide a useful framework for this determination?

The framework is logical, but I am not sure about how practical it will be. As indicated below, addressing some of the Hill criteria in any sort of comprehensive way with existing data sets may be a problem for many agents.

c. Is the document clear, complete and objective?

The general steps in the process are listed in section 2.0, and there is a more detailed outline (or a set of bullets) for applying the MOA framework in 2.4. The essence of the process is two evaluations: in the simplest case, first deciding if a carcinogen is a mutagen, and if it is a mutagen, deciding if its tumors are induced via a mutagenic MOA. The two decisions (WOE determinations) are explained in sections 2.3 and 2.4 and their following sections and subsections, respectively. The WOE process for mutagenicity determination is similar to other similar evaluations. The only bone I had to pick with this section was that it left the impression that a positive response in e.g. a bacterial mutagenicity assay would somehow be mitigated by a negative in cyotogenetic assays (through WOE). This ignores the principle of the battery approach to genetox testing: that no single assay can detect all possible genetoxicity effects. A positive in one test of a battery indicates mutagenic activity, whether or not another test is negative. This is acknowledged at the beginning of section 2.1 but seems to be lost in section 2.3 and its subsections. Or maybe this is all contained in the principle of professional judgment.

I think that the whole process section could be structured better. Section 2.0 spells out the major steps nicely; however, this nice arrangement gets lost in the subsequent sections. In particular, section 2.4 was a problem for me. The process for the mutagenic MOA determination is: formulate an hypothesis that includes a description of the key events, and determine if the data support mutation as a key event for cancer, essentially by applying the modified Hill criteria to the data to establish causality, then make judgments as to whether or not the data support the hypothesis for a mutagenic MOA in animals, and then in humans. This is essentially the same procedure as is used for MOA in the Cancer Guidelines, and it is spelled out in bullets in section 2.4. But after the bullets the section goes on to provide a 'framework' (pg. 20, l. 18) for what sorts of data may be useful in the determination, and indications of what impact that specific data would have on the WOE. The framework is rather general, with disclaimers, and there may be a tendency to focus on the specific examples of information that are provided, even though it is characterized as not a check list. My major problem with this section is that it separates the introductory bullets from section 2.4.1 and beyond, which go on with explaining the process step by step. I would

suggest that p. 10 l. 23 to p. 11 l. 19 should be placed in the section dealing with strength, consistency, and specificity of association. Also, section 2.4.3 dealing with site concordance, does not describe one of the Hill criteria, and rather than break up the logical progression of the process, this could also be folded into the subsection on strength, consistence, and specificity. Also giving equal weight to all the subsections within section 2.4 does not help clarify the process of determining a mutagenic MOA. This arrangement I found a little confusing and I think can be improved upon.

Perhaps something like this would be clearer:

1. Introduction
    a. …….
    b. …….
    c. ……
    d. ……..
2. Collect the data relevant to the MOA determination
    a. Assemble data useful for establishing the intrinsic mutagenicity of the agent (usually from standard battery assays or follow-up assays used for hazard ID)
    b. Assemble additional data relevant to mutagenicity (including DNA adduct, ADME data and nonstandard assays used for the mutagenicity WOE plus additional data; part of section 2.4.9?)
    c. Assemble data on agent carcinogenicity and other possible MOAs (important for dose response concordance and temporality assessment)
3. Evaluate assembled data against acceptance and quality standards
4. Use the data to judge WOE that the agent has mutagenic acitivity
5. If mutagenic, apply the MOA framework to determine if the agent has a mutagenic MOA for cancer.
    a. Generate an hypothesis for a mutagenic MOA that describes the key events
        i. Describing carcinogenesis in terms of key events
        ii. Observations supporting mutation as a key event
    b. Test the hypothesis by applying the modified Hill criteria to the data
        i. Strength, consistency ect. (including site concordance, section 2.4.3)
        ii. Dose-response concordance
        iii. Temporality
        iv. Biological plausibility and coherence

c. Consider the plausibility of other MOAs
d. Identify uncertainties, ect.
e. Make conclusions on the proposed mutagenic MOA
    i. Is the mutagenic MOA supported in animals? (this section could be improved)
    ii. Is the mutagenic MOA supported in humans?

While the document seemed to want to keep the message general, and avoid specifics that might back regulators into a corner, some detail was included and it was not always clear why it was (a section on QSAR, other uses of genetox data, EPA genetox batteries), and other detail was omitted or treated unclearly (what was the message on the target organ specificity—important or not?).

d. Are major limitations and assumptions clearly defined?

Yes; multiple caveats and references to the limited application of the document are given beginning in the Preface.

2. Given the inconsistency in the scientific community of genetic toxicologists regarding the definition of mutagenicity, the *Framework* (section 1.4) proposes an *operational* definition of "mutagenicity" for a very limited use, i.e., determination of a mutagenic MOA *for carcinogenicity* under the U.S. EPA *Cancer Guidelines* and *Supplemental Guidance*. Please discuss whether this definition is useful and appropriate, considering the limited application in this *Framework*.

I suspect this definition is a point of contention whenever these documents (this Framework and the previous Guidelines/Guidance) are discussed or this wouldn't be a part of the 'charge'.

There may be some genetic toxicologists who don't agree on the definition of mutagenicity, but then again in a large group you can probably find a few individuals who don't agree on a lot of things. The strange thing is that the Framework isn't consistent about its definition of mutation and mutagenicity. Most genetic toxicologists that I know use the same definition of mutation and mutagenicity: mutation is a change in DNA sequence that can be inherited through successive generations; mutagenicity is the capacity to induce mutation. This is contained in the Framework definition on pg. 8, l. 7, but it is contradicted by the definition from the Supplemental Guidance that is quoted on pg. 9, l. 7. The problem here is that chromosome aberrations (CAs), as measured in standard CA assays, and most micronuclei

(MN) are not mutations because they are mostly acentric fragments and cannot be inherited. Stable chromosomal mutations (e.g., translocations and interstitial deletions) are hardly ever recognized in these assays and aneuploidy (which, along with clastogenicity, is easily detected in MN assays) is classified by the Framework as not due to mutagenesis. The mixed message is compounded in Appendix A where CAs and MN are detected in vivo by chromosome damage assays and in vitro by chromosome mutation assays. I suspect that the intent is to use the Supplemental Guidance/Guidelines definition, so these discrepancies in the Framework should be cleaned up. I understand from previous discussions with EPA people that this definition was agreed upon for the Cancer Guidelines/Guidance, and, right or wrong, this is what EPA is sticking to.

There is another change from the common definitions of mutation and mutagenicity. The distinction here is that the binding of a test compound or its direct metabolite is necessary for inducing a mutation. Although this distinction classifies a lot of mutations as not being due to mutagenicity (which seems very odd), it has the practical value of providing a rationale for excluding mutations produced by oxidative compounds and spindle poisons as being due to mutagenicity. In the context of the document, I suppose that this is at least useful.

3. **Section 1.2 (uses of mutagenicity data) was added in coordination with and at the request of other Federal agencies that wished to distinguish their use of such data from the use in this *Framework*.**
    a. **Is this section clear and sufficiently complete to accomplish that goal? Please suggest recommendations to improve this section.**
    b. **If this section is not complete, what other specific uses of mutagenicity data for regulatory purposes might be addressed?**

This section seemed odd to me when I first read through the documents. For the purpose of this document, do we really need to know how else mutagenicity data are used? But if others want it there, it can do no harm. As far as regulatory uses of mutagenicity data, it seems complete.

4. **Which version of Figure 1 best captures the steps proposed in the *Framework*? Please identify the basis for your recommendation.**

I like version 2 better, with reservations. I think it useful to indicate that there is a possibility that that the data may be insufficient to determine if an agent is mutagenic or not, and that there may be insufficient

information to determine if a mutagenic agent has a mutagenic MOA for a particular tumor or not. I don't think this possibility (i.e., can't tell) is clearly delineated in the text (only towards the end, p. 28, l 2?), and it should be. I do disagree, however, with some of the placements of the arrows in the figure. There is also a difference between the figures on agents whose mutagenicity cannot be characterized. In the first version these agents are considered for a nonmutagenic MOA, in the second they are put in the 'can't determine a MOA' box. Not sure which is correct.

I also don't see how the table following the chart in the second version fits into the context of Section 2.0. The WOE referred to in the text section is for determining whether or not an agent is mutagenic (covered in more detail in Section 2.3.1), not a WOE for whether or not mutagenicity is a key event for a particular tumor (which is the subject of the table). This step is not referred to as a WOE in the section and there is only an implied reference to it in step 4. Perhaps it can be referred to directly somehow in this section. It seems to fit better with section 2.4 and later, which allude to the importance of in vivo data (although not to the degree indicated in the table and without breaking down the various kinds of in vivo data as is done in the table.) Although it is an interesting way of weighing genetox data relative to tumor induction, from the information in the text, it is not clear how this WOE is used in the Framework (in a particular step in evaluating the Hill criteria?). See additional comments on this below.

5. **The MOA framework is defined in the *Cancer Guidelines*. The steps are repeated in the *Framework* with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.**

    a. **Is the information in this *Framework* on the application of the *Cancer Guidelines* MOA framework sufficiently clear and complete to be useful?**

    I think so. The Framework repeatedly refers back to the Guidelines and its application of the Hill criteria is consistent with the Guidelines. If other frameworks were written to cover possible nonmutagenic MOAs (e.g., epigenetic), I suspect the same approach could be taken.

    b. **Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data that are generally available for consideration? If not, what additional elements might be useful to include in the *Framework*?**

    It does but in a very superficial/general manner, but I suspect this is the intent. As indicated below, the ultimate success in applying the elements of the Framework may be another issue.

c. Please review each step, e.g., "key events," "dose-response relationships," "temporality," and discuss what types of additional information might be added (remembering that this document can not suggest or require additional testing). Given the current state of science, please comment on the application of each step in the MOA framework.

The weakness of applying the Hill criteria to determining a mutagenic MOA is that there are probably little existing data addressing dose response concordance or temporality for most agents. These data most likely have to be in vivo data and most in vivo experiments are set up as hazard ID assessments. The experimental protocols recommended by the IWGT and OECD for the various in vivo assays also are intended for hazard ID and are probably not ideal for MOA determination. Most genetox experiments do not use the treatment conditions used in cancer bioassays (presumably the best way to make comparisons between genetox and tumor endpoints), and as stated in response to question 14, I am not sure how useful dose response concordance will be for genetox data, even in a general way. I think temporality data may be useful but most studies do not sample over a period of time, as would be necessary to assess temporality. In addition, most cancer bioassays do not have intermediate sacrifice times and/or look for the timing of preneoplastic lesion development. As stated on the bottom of pg. 22 and on pg. 23, l. 27, mutation is probably a 'key event' for a lot of tumors, including some induced by agents with EPA's definition of a nonmutagenic MOA. The timing of the mutation (early and as a direct result of agent binding to DNA), is a major consideration in determining a mutagenic MOA. Perhaps publishing this Framework will cause people to rethink how they design experiments, but for the near term I suspect data addressing dose response concordance and temporality will be few and far between. Will hazard ID data be sufficient to make a call on mutagenic MOA, or put differently, are dose response concordance and temporality data necessary? If not, there will be a lot of agents in the 'can't decide' box of Fig. 1.

6. Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader? Does it adequately describe how the quality of the data can be evaluated? If not, how could it be expanded or improved? Please provide specific recommendations.

I think this is a useful section. Older (and some newer) published data may be quite misleading if the assays were performed in an inadequate manner, and some judgment should be made as to the quality of the data. I think that the sources that are listed for testing guidelines are good, although at least some of the particular citations are overview/review papers, and not current guidelines for individual assays. I think that the specifics of protocol design and adequate data analysis are spelled out pretty well by the publications from the individual organizations and need not be gone into in any detail here. If the EPA wants to be more specific for a particular assay, they should construct a table and cite particular guidance documents for that assay. One problem for this is that these guidelines for data acceptance and quality are typically for hazard ID assays, whereas important data addressing cancer MOA (as indicated above) may not conform in all details to how hazard ID assays are run.

7. **Are the discussions of aneuploidy adequate? If not, how should they be expanded? Please provide specific examples and detailed information regarding recommendations.**

The classification of aneuploidy is an interesting one since some aneuploid cells have a stable change in DNA sequence, and thus fit the common definition of mutation. And it is logical to suspect that mutations are induced due to the 'mutagenicity' of an agent (or endogenous process). However, because this mutagenicity may not be caused by the interaction of the test article (or its direct metabolite) with DNA, this may be one instance where EPA's definition reclassifies a mutagen to a nonmutagen. Is it always the case that aneugenicity will be due to interactions with spindle fibers, or at least primary interactions that do not involve binding to DNA? This is the implication of the statement on pg. 22, l. 6, for instance. I can imagine instances where DNA damage might result in chromosome loss, although I don't recall any strong evidence for this (but I am not a cytogeneticist). Is the assumption implicit in identifying an aneugenic response (e.g., through kineticore staining) that it is not due (even partially) to mutagenicity (under the Framework definition) or is some additional evidence required to exclude DNA damage or to demonstrate protein binding as the sole mechanism? It might be helpful to explain this more clearly.

8. **Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutational event, or knowledge of mutational spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.**

I think that this knowledge would add to the WOE assessment connecting the mutagenicity of an agent (determined in the first stage of the analysis in Fig. 1) to MOA for tumor induction (the second stage in

Fig. 1). It seems to address the bullet point starting on 24, l. 30. If, for instance, there was evidence that the DNA damage produced by the test agent resulted in a high frequency of G>T transversions, and that G>T transversions were found in a reporter gene in the target tissue, or better yet, CAA>AAA was found in H-ras codon 61 from liver tumor DNA, or G>T was found in p53 from lung tumors, yes, I would say that this is (strong) evidence that the mutagenicity of the test agent is responsible (i.e., a key event) for the tumor induction.

9. **Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.**

This would seem to fall into a policy/management decision for proceeding with a MOA determination for a particular agent. If a determination were reached on the basis of weak evidence, this could be noted and the agent's classification revisited when appropriate. Also, according to Fig. 1, one of the outcomes of a MOA evaluation is 'Unable to determine…'. It is a policy decision as to whether or not it is worth the effort when the likelihood of reaching such a conclusion is high. It depends on how high the bar for burden of proof is set (another policy decision) as to how many agents fall into the 'can't determine' category.

10. **The WOE for determining an MOA for carcinogenicity is described and determined in the U.S. EPA *Cancer Guidelines*. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss if we have achieved this goal. If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?**

I like the reliance on the Hill criteria for determining cancer MOA: it seems like a very logical approach to address the question without be too prescriptive of the details of how it will be accomplished. Determining a MOA, rather than a mechanism of action, seems to be able to leave the details of the process in a black box, providing a lot of flexibility in how the ultimate decision is made. Acknowledging that knowledge about carcinogenesis is evolving and having the document respond to the changes also provides flexibility. The problem may be in practical application of the Guidelines (as stated elsewhere in these comments).

11. **For groups of similar compounds, e.g., nitrosoamines or polycyclic aromatic hydrocarbons (PAHs), could a mutagenic MOA be supported solely by structure activity relationships or analogy? Apart from the consideration of groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by structure activity relationships or analogy? If not, what additional information would you think might be needed?**

This is more of a policy decision than a scientific question. As a bench scientist, I would like to see at least some experimental data that confirm the predictions. But predictive models (e.g., SAR models) have an error rate that is knowable for a particular class of compounds, so the uncertainty in the decision is knowable. I would be less comfortable with a seat-of-the-pants decision that lowers the level of concern for an agent. Science is full of surprises; when I make informed guess conclusions like this I am often wrong. But EPA is probably smarter than I am!

12. **Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (Section 2.4.9) and in humans (Section 2.4.10).**

I thought the human section was OK. If the tumor oncogene/suppressor gene mutations are consistent with the mutagenic specificity of the test agent, I would think this would be evidence for a causal relationship.

Since there will probably be more in vitro and animal cell data to consider, I thought that guidance in how to use the data would be a little more detailed. The section makes a vestigial attempt to do this, but not much. Perhaps the table from Fig. 1 would be useful here?

13. **Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.**

I am not completely sold on the necessity of the Appendices. I thought Appendices B-D were interesting, but not necessary. The table in Appendix A seemed obvious; if retained, it might benefit from an additional column on whether or not the data conform to current guidelines for the assay. Also, remove the repeated in vivo section, and consider whether or not CAs and MNs are mutations (see above).

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

There are some agents, perhaps more than are now recognized, that produce DNA damage simultaneously by both mutagenic and nonmutagenic mechanisms. Take the classical mutagen UV, which forms dimers by the direct deposition of energy (mutagenic MOA), and oxidative lesions by the generation of oxidative species (nonmutagenic MOA). The possibility of these 'mixed' MOAs is not addressed by the Framework, and whether or not it is important to parse out the biological effects attributed to each.

I think that it would be relatively difficult to totally rule out mutation as a key event in tumors induced by a mutagenic agent. Even if there was evidence for a nonmutagenic MOA, given that all assays have a limit of sensitivity and that there is no consistent quantitative relationship between simple measurements of mutation, chromosome aberration, adducts, ect., how can at least a mixed MOA be ruled out? Most common genetox assays are designed for hazard ID, not for quantitative risk assessment.

This Framework and the Cancer Guidelines assert that agents that produce tumors at multiple sites (or presumably different types of tumors at a single site) may have different MOAs for the different tumors and that a general approach is presented to determine these MOAs (e.g., p. 10, l. 6; p. 23, l 16). I see very little in the Framework indicating how tumor sites or tumor types are treated individually for determining a mutagenic MOA, other than passing references to site specific genetox data (p. 23, l 32, p. 28, l. 15). Is this how it is done? The Guidelines and Framework both mention determining MOAs for individual tumors as important so it appears that it has some significance for the risk assessment process. Is this the level of clarity that EPA intends?

An implied message from the document is that it may be difficult to conclude a mutagenic MOA without additional data showing that mutation occurs early in the process and precedes other possible nonmutangenic key events. In other words it will take more than genetox data to establish a mutagenic MOA. I guess this is stated in pg. 23. l. 11, but not very clearly.

Charge question 5c indicates that the judgment will be made with the available data and additional data will not be requested. I couldn't find this in the document, but I could find passages that indicate where additional testing may be necessary (pg. 21, l. 27). This seems somewhat inconsistent.

It says on pg. 20 and 21 that in vivo data are important to making this decision but it never is clearly stated what kind of in vivo data are important and which are more important. The WOE table in Fig. 1 tries to rank order the data, and I have no argument with the ordering, but this does not appear to be used anywhere in the process described in the text. It seems that whoever wrote the text was willing to go as far as indicating that in vivo data are very important, while the person(s) who put Fig. 1 together felt more detail was necessary, but the two intentions were never fully integrated into the Framework.

The abbreviation, MOA, is used inconsistently throughout the text.

P. 10, l. 28: says the genetic tox data used for hazard ID testing schemes describe the range of data that may be used for determining a mutagenic MOA (not only mutagenicity). This is a little misleading in that other genetox data (as well as other types of data) can be useful for the mutagenic MOA determination. But the next sentence says assemble all possible relevant data (not only data from standard battery assays but including data from major toxicity databases, SARs, pharmacokinetics). Is all this consistent? Shouldn't this section just deal with data for the mutagenicity assessment, not also the MOA assessment? It would be simpler to take one step at a time (see my suggested arrangement in charge question 1).

P. 15, l. 11: Shouldn't 'below' be 'above'?

P. 16, l. 18, Change 'of' to 'using'.

P. 18, l. 10, Change 'decides' to 'decide'.

P. 21, l. 11: Substitute 'increases' for 'strengthens the support for'.

The bullets starting on the bottom of p. 21 and on pg. 22 l. 10 don't make any sense to me.

Section 2.3.2: this is a little cryptic: either a compound is a mutagen or it isn't. I don't understand how it can be mutagenic in one context and not in another using the same datasets.

The paragraph on pg. 23, l. 11 could be a little clearer.

Pg. 24, l. 12: but aren't initiators in initiation-promotion experiments usually used at concentrations at which they are not complete carcinogens?

Bullet pg. 24, l. 30: I am not sure what this means.

P. 25, l. 28, ‘mutagenicity’, not ‘mutagenic’.

Pg. 25, l. 31: But if an agent produces genetox responses in various tissues, but not in the target tissue, I would think that this would not support a mutagenic MOA.

P. 31, l. 13: Change ‘is’ to ‘must be’.

P. 46, l. 12: Add close parenthesis after ‘2003’.

The level of proof necessary for assigning a mutagenic MOA is, probably by design, kept vague. However, pg. 28 l. 37 implies that a positive in vivo MN assay would almost always result in this conclusion. Is this the intention? Or does ‘operative’ mean that a mutagenic MOA should be considered? Perhaps this should be clarified.

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Response To Charge Questions on EPA's Document, "Framework for Determining a Mutagenic Mode of Action for Carcinogenicity:

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Responses to Charge Questions.

Introduction

Background

Page 5, paras. 1-3: This is a good discussion, and orients the reader to the relationships between this document and the Guidelines for Carcinogenc Risk Assessment and the Supplemental guidance for assessing susceptibility from Early-Life Exposure to Carcinogens.

1. Charge Question #1:

a. Is the purpose for this document clear?

Yes, the purpose of this document as described in the Preface on page 4 is clear. This document is intended to help EPA risk assessors determine whether data support a mutagenic mode of action (MOA) for carcinogenicity of chemicals in general. This document was written because EPA's 2005 Guidelines for Carcinogen Risk Assessment emphasize using MOA information to interpret and quantify potential cancer risk of chemicals to humans. Hence, this document clarifies using MOA for mutagenic carcinogens in risk assessment. In addition, this document is valuable because the Supplemental Guidelines recommend that age-dependent adjustment factors (ADAFs) be used with the cancer slope factors and age-specific estimate of exposure in development of risk estimates if the weight of evidence (WOE) supports a mutagenic MOA for cancer induction. Because of the broad impact of judging an agent to have a mutagenic MOA, EPA's risk assessors need to approach identification of a mutagenic MOA for carcinogenicity in a consistent and scientific manner. Hence, yes, the purpose of this document is very clear. This document basically expands and clarifies discussions found in the Cancer Guidelines and Supplemental Guidance on how to evaluate data to determine whether or not a chemical has a mutagenic MOA for carcinogenesis.

b. Does the document provide a useful framework for this determination?

In general, yes, this document does provide a useful framework for this determination. I have mentioned two areas that do need to be addressed,
1) that many carcinogens have mixed modes of action (ex: insoluble nickel compounds, arsenic compounds) or have modes of action that after many decades of research have not been elucidated; and 2) that if a compound is mutagenic, if its MOA is not completely established, in the absence of strong evidence that it has a non-mutagenic MOA, it should be dealt with by risk assessors as if its carcinogenicity followed a linear, no threshold dose-response curve. Further, in my opinion, the risk assessors should not depart from the default assumption of a mutagenic MOA and a linear dose-response curve, unless there is strong evidence to the contrary for a non-mutagenic MOA.

c. Is the document clear, complete, and objective?

The document is written in a fairly clear fashion. It is a fairly complete document. However, some discussion of chemicals such as the insoluble nickel compounds NiO/NiS/nickel subsulfide and of arsenic compounds, which likely have mixed MOAs, should be made. Some additional discussion of using the default approach for a linear, no-threshold dose-response curve for chemicals whose MOA is not know at all, or whose MOA may be mixed and include mutation as part of the mechanism (i. e., insoluble nickel compounds, arsenic compounds) should be mentioned and discussed briefly.

d. Are major limitations and assumptions clearly defined?

Yes, most of the major limitations and assumptions are clearly defined in this document. There was an ample discussion of the limitations of the data bases for carcinogenicity and mutagenicity for chemicals in general.

2. Charge Question #2: Is the operational definition of "mutagenicity" for a very limited use, i. e., determination of a mutagenic MOA for carcinogenicity under the U. S. EPA Cancer Guidelines and Supplemental Guidance, useful and appropriate, considering the limited application in this Framework?

Page 8, para. 4: I disagree with these definitions. This paragraph mixes up "mutagenicity" and "genotoxicity." It is appropriate to define genotoxicty broadly, and to define mutagenicity as a subset of genotoxicty, and to define mutagenicity broadly within its definition, including aneugenicity as a subset of mutagenicity. However, I would consider unscheduled DNA synthesis and sister chromatid exchange under genotoxicity, not under mutagenicity, because there is no strong evidence that they are necessarily mutagenic events, although they are clearly genotoxic events. Please tighten up the terminology here, and make it applicable to what is conventionally used in the Genetic Toxicology Community.

Having said this, I find the definition on page 9, para. 3, appropriate for classifying a chemical as a mutagen. In this paragraph, I would broaden definition to state, "Key data for a mutagenic mode of action may be evidence that the carcinogen or one or more of its metabolites can react with DNA and/or has the ability to bind covalently to DNA (e. g., PAHs, aromatic amines, nitrosamines) or to bind coordinately to DNA [e. g., cisplatin, Cr(VI)], or to generate reactive oxygen species and oxygen radicals (superoxide anion, hydrogen peroxide, hydroxyl radical), that can cause damage and resultant mutations to DNA. " It is important to be precise and also broad here to capture all the situations of mutagens.

On page 9, para. 5, I recommend adding the following sentence at the end of this paragraph: "However, if a chemical carcinogen has been shown to be mutagenic, in the absence of other strong and conclusive data showing a non-mutagenic MOA for carcinogenesis, this chemical will be presumed to have a mutagenic MOA. For risk assessment purposes, the risk assessment procedure for such a chemical will be conducted according to a default procedure in which a linear, no-threshold dose-response for carcinogenesis will be presumed to be operant. This chemical will then be regulated as a mutagenic carcinogen with a mutagenic MOA."

3. Charge Questions #3: Regarding Section 1.2 (Uses of Mutagenicity Data):

a. <u>Is this section clear and sufficiently complete to accomplish the goal of distinguishing the goal of other Federal agencies that wished to distinguish their use of such data from the use in this Framework?</u>

Yes, in general, this section is clear and sufficiently complete. As such, this section does delineate the goal of other Federal agencies to distinguish their use of such data from the use in this Framework.

I have one specific comment here: On Page 6, para. 4, point #1: On lines 8- 9, I recommend changing this sentence to read, would change this sentence to read, "…to predict the likelihood of adverse outcomes, such as cancer induction, in the absence of information in animals or humans on this outcome."

b. **<u>If this section is not complete, what other specific uses of mutagenicity data for regulatory purposes might be addressed?</u>** :

My opinion is that this section is clear and sufficiently complete.

4. Charge Question #4: Which version of Figure 1 best captures the steps proposed in the Framework? Please identify the basis for your recommendations.

I like figure 1 v2 better. It is more comprehensive and more clear. However, I would recommend adding, for the middle path, "Data Unavailable or insufficient to determine if chemical can induce mutation, or if a mutagenic chemical carcinogenic has a mutagenic MOA." Then add another box, which says, "Mutagenic Chemical without a conclusive MOA for cancer is regulated by the default procedure as a carcinogen with a mutagenic MOA (unless other non-mutagenic MOAs can be substantiated)."

5. Charge Question #5: The MOA framework is defined in the Cancer Guidelines. The steps are repeated in the Framework with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.

a. <u>Is the information in this Framework on the application of the Cancer Guidelines MOA framework sufficiently clear and complete to be useful?</u>

Yes, in general, it is sufficiently clear and complete to be useful. However, I strongly recommend adding that if a mutagenic chemical carcinogen does not have a conclusive mutagenic MOA, but no other evidence exists to conclusively document a non-mutagenic MOA, then this chemical is treated as acting by a mutagenic MOA for risk assessment and regulatory purposes. For risk assessment purposes, this chemical is presumed to follow a linear, no threshold, dose-response curve for cancer induction."

b. **Does the Framework provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amounts of data that are generally available for consideration? If not, what additional elements might be useful to include in the Framework?**

Yes, in general, the Framework does provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amounts of data that are generally available for consideration. However, I note that there are many complexities to the process of carcinogenesis. For instance, PAH bind covalently to DNA, cause mutations and chromsome breakage, and also are thought to generate oxygen radicals and even to alter DNA methylation. Scientists have been studying the MOA for PAHs since l900, and still complexities arise as research continues. The MOA for PAH is presumed to be mutagenesis, but there are many complexities. I recommend being circumspect and acknowledging these complexities in risk assessment to make this document as academic and appropriately scientific as possible.

Similarly, for insoluble nickel compounds, these are very complex reagents with a very complex, almost certainly dual, MOA for carcinogenesis. They are genotoxic and mutagenic (they cause chromosome damage, induce micronuclei, and cause gene amplification, and they also cause epigenetic/non-genotoxic events – induction of methylation of tumor suppressor genes. Hence, they almost certainly have a dual MOA – mutagenic/genotoxic and also non-genotoxic/non-mutagenic (methylation effects). Hence, I recommend acknowledging this complexity for this type of chemical carcinogen, and also recommend using a linear, no threshold dose-response curve for risk assessment and carcinogen purposes, unless and until it can be conclusively sorted out as to whether and which mechanism, genotoxic or non-genotoxic, predominates at the lower concentrations of this reagent (nickel subsulfide, black nickel oxide, green nickel oxide, crystalline nickel monosulfide, etc.). Further, what would you do for risk assessment if, as I suspect, these compounds act by a mixed MOA?

I also recommend mentioning arsenic compounds as very complex reagents which can generate oxygen radicals and cause clastogenesis, and can also cause changes in DNA methylation. I would posit this type of carcinogen as one which should be labeled "genotoxic/mutagenic," but its risk assessment done by a linear, no threshold dose-response curve, because its exact MOA has not yet been conclusively defined. For some chemicals, it will likely take decades to ascertain the MOA with certainty. For some, we may never reach scientific consensus on what the MOA is for carcinogenesis.

Some discussion of why the risk assessment for Trichloroethylene has never been finalized, likely due to its many complex actions on cells and tissues, might be instructive. Perhaps such material can be added to another appendix, to keep the original document concise.

c. Please review each step, e. g., "key event, "dose-response relationships," "temporality, " and discuss what types of additional information might be added (remembering that this document can not suggest or require additional testing).

Key Event: The discussion here is pretty good. Analyzing how the data support the key evens and how other relevant associated data support the hypothesized mutagenic MOA is appropriate. It is appropriate also to analyze all available information on cellular interactions, for parent chemical and its metabolites, and to analyze the absorption, distribution, metabolism, and excretion (ADME) aspects of a chemical exposure. Studying the physiological, cellular, and biochemical differences among species by PBPK and evaluating the toxicodynamics and consequences of the interactions between the chemical/-metabolite and the target cell, tissue, or organ is also appropriate. Analyzing the strength, consistency, and specificity of association between genetic events and outcomes is also appropriate. Similarly, analyzing the dose-response concordane, discussing the temporal relationship, and analyzing the biological plausibility of coherence of the database is also fine. However, it is important not to bog the professional risk assessment scientist with too many considerations. These should be guidelines that he/she may check during the conduct of a risk assessment. Too much focus on these points would prevent risk assessments from ever being completed.

Under key event, I recommend a short discussion that we now know from the work of Vogelstein and collaborators, that there are approximately 15 mutations that are considered important in the genesis of tumors. These 15 mutations correspond with a parallel differential expression of approximately 150 genes, some increased in expression, some quiescent in expression. Hence, each mutation leads to a further 10 genes to be over-expressed, if it occurs in a cellular proto-oncogene, which resides in a signal transduction pathway, or leads to a further 10 genes to become quiescent in expression if the mutation inactivates a tumor suppressor gene, which controls the expression of an additional ten genes, such as by acting as a transcription factor. Hence, there are profound effects on tumor cells at the level of gene expression.

On page 24, line 1, the authors state: "DNA of the target cellor tissue is damaged." This is a genotoxic event. This is why I recommended broadening the definition of this document to "genotoxic," rather than limiting it to "mutagenic."

Dose-Response Relationship: This is one of the most important steps in the risk assessment process. In general, this section is written very well.

Temporality: This section is written in an appropriate manner.

Given the current state of science, please comment on the application of each step in the MOA framework.

6. Charge Question #6: Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader?

Yes, this section is transparent to the reader.

Does it adequately describe how the quality of the data can be evaluated?

The section is pretty terse. However, I do really recommend that the professional risk assessment expert use his/her judgment, and not be bogged down by too many rules.
Otherwise, the risk assessment will never be completed. Hence, this section is fine as it is.

If not, how could it be expanded or improved? Please provide specific recommendations.

You might consider giving a few examples, if you have the space in the document and time to do this, or else place them in the Appendix.

7. Are the discussions of aneuploidy adequate? If not, how should they be expanded?
Please provide specific examples and detailed information regarding recommendations.

8. Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutation event, or knowledge of mutation spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action?

Yes, in my opinion, knowledge of the type of mutational event, and knowledge of the mutation spectrum, both contribute to the WOE for a mutagenic mode of action. Both sets of data are very important. Certainly, for the nitrosamines, animal experiments showed that they cause a G/C to A/T type of mutation in ras genes, and this is the exact type of mutation that nitrosamines cause in other in vitro systems – bacteria, mammalian cells. This is very useful data, and leads to a concordance that is useful in determining a mutagenic MOA. In other cases, such as aflatoxin B1, which causes mutations in the p53 gene in liver cancer, determination of the mutational spectrum is very useful in substantiating that indeed, aflatoxin B1 caused this cancer, and did so by a mutagneic MOA, because the mutational spectrum is different from the mutational spectrum of spontaneous tumors. Hence, both types of data are important where they are available.

Please provide specific recommendations for using either the mutational event or mutational spectra.

As discussed above, I would use both of these types of data where they are available. They are both precious and important types of data that can help lead to the conclusion of a mutagenic MOA for carcinogenesis.

9. Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive data base is not available.

Of course, one treads carefully when the data base is not extensive, and deciding on whether a mutagenic MOA exists is difficult. If there is no in vivo data for the chemical of interest, one is then pretty much stuck with using any in vitro data that exists. If there is a sufficiently robust in vitro mutagenesis data base with numerous positive results, it is not too much of a stretch to designate a chemical as acting by a mutagenic MOA, tentatively. In this case, I would recommend using a linear, no threshold dose response curve fit according to a default assumption, and this is appropriate, conservative,

and protects the health of the public. It can be a tentative solution until more in vivo data is developed for that chemical.

In case 2, where substantial in vivo and in vitro data are available on the mutagenicity of a structurally similar group of chemicals (or where, appropriate, their reactive metabolites), I am less confident. In such a case, one is relying on an extrapolation, not on actual chemical itself. In this case, I would recommend applying a default assumption tentatively, until a larger in vitro and in vivo mutagenicity data base for this chemical materialized. This situation will likely become common, due to the large numbers of chemicals that need to be regulated, and extensive data bases on some of their congeners. If the chemical was important commercially, I would recommend that the NTP took a hand in generating in vivo and in vitro mutagencity data rapidly, or that contracts be advertised from NCI/NIEHS/NTP for academic researchers to conduct the necessary work and to obtain the necessary data.

In case #3, where information on the toxicokinetics of the chemical of interest support formation of the reactive species, this again is a situation that makes me nervous in terms of risk assessment and regulation. One can be fooled if the reactive species is formed, but detoxified by phase II conjugation. Probably in such a case, it is appropriate to use a default mutagenic MOA, but to continue to gather data on the chemical in terms on in vivo and in vitro mutagenicity assays, until the issue of a mutagenic MOA is resolved conclusively one way or the other.

. .

10. The WOE for determining an MOA for carcinogenicity is described and determined in the U. S. EPA Cancer guidelines. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss whether we have achieved this goal.

If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the Cancer Guidelines?

Overall, I believe that you have achieved the goal of describing a framework for organizing data, determining relevance of those data, and considering issues on a mutagenic MOA for cancer. However, I do have some suggestions and recommendations that could help improve your efforts, discussed below.

On pages 7-9, the authors discussed what an MOA for mutagenicity would entail, and discussed mutagenicity in the context of a mutagenic MOA for Cancer. This discussion was clear. On pages 10- they discussed evaluating the data against current acceptance and quality criteria, and judging the weight of evidence (WOE) that the chemical has mutagenic activity. The authors further discussed categorizing the data, and describing the WOE for Mutagenicity. They noted that the decision that the chemical is mutatgenic will be based on the overall WOE. They appropriately discussed the meaning of the terminologies "inconclusive" and "contradictory." This discussion was appropriate. They discussed Morphological Cell Transformation and In vivo Spermhead Abnormality Tests. I felt that they could have done a better job of discussing Morphological Cell Transformation Assays. They should note that these assays are the closest one can come to studying tumorigenesis in animals, and that these assays detect both genotoxic and non-genotoxic carcinogens by their ability to induce morphological cell transformation. Mention could be made of the currently used test systems – C3H/10T1/2, Balbc 3T3, Syrian hamster embryo cells – as used with and without exogenous S-9 metabolic activation. A few references could be placed here describing these tests for morphologically induced cell transformation.

The section on Evaluating Results Across Endpoints was well-written. It was very good that the authors stated that "All WOE conclusions depend on professional judgment; these judgments are discussed in a clear and transparent manner."

Page 17,last two lines and continuing on to page 18: I recommend stating this sentence in a more positive fashion, as follows: "Similarly, positive results in cell transformation assays may be associated with chemical carcinogens, both with those that have a mutagenic MOA, and also with those that act via a non-mutagenic/non-genotoxic MOA."

I disagree with the sentence on page 18, para. 1, lines 3-5: I would recommend stating this sentence as follows: "Positive results in only the cell transformation assays likely indicate a non-mutagenic mode of action. Positive results in the aneuploidization test and the cell transformation test only likely indicate that aneuploidy is an MOA for carcinogenesis, and since aneuploidization is a mutagenic event as far as the cell is concerned, this would indicate a mutagenic MOA, with mutagenesis defined broadly and including gain or loss of chromosomes by the cell.

Under the section, 2.3.2.3, WOE Conclusions for Mutagenic Activity, I note that this section is well-written and informative. On page 18, para. 4, bullet point 1, I would suggest changing this statement, to the following: "The data are sufficient for a judgment of negative. The chemical has been tested in acceptable studies, and all or very close to all, of the acceptable assays are negative. Any few positive studies in this data set do not indicate specific genotoxicity in a specific assay system, but are due to random variation and noise in the biological system." This would guard against ignoring positive tests in one specific system, and calling this a negative result overall, when all the other tests were negative, but the results in this one specific test were positive.

For the reasons just discussed, please be more careful in describing an equivocal data base for mutagenicity. I am worried that inexperienced risk assessors would look at this and conclude that if a chemical is positive in one test and negative in the other tests, that it is negative, which would not be true. I have run into this situation on toxic air contaminant review boards.

11. For groups of similar compounds, e. g., nitrosamines or PAHs, could a mutagenic MOA be supported solely by structure activity relationships or analogy?

Certainly, for PAHs, the use of the Bay Region Theory of Dr. Donald Jerina of NIH could be very useful in itself to help support a mutagenic MOA based on calculated and predicted formation of a bay region diol epoxide, which can subsequently generate a carbonium ion in the bay region, which would lead to subsequent binding of the PAH diol-epoxide carbonium to DNA, mutation, and cancer. Since these events are imporartnt in generating an initiator for cancer from PAH, positive quantum mechanical calcuation results in stability of formation of the bay-region diol-epoxide carbonium ion jwould indeed predict two things: 1) that this specific PAH is a carcinogen, and 2) that it is a mutagen, and thereby likely acts by a mutagenic MOA. In fact, this theory can predict that BeP, the isomer of BaP, is a non-mutagenic, non-carcinogen, whereas BaP is a mutagenic carcinogen. This does require some quantum mechanical calculations, but these can be completed rather quickly by an experienced physical chemist. Hence, yes, this can be done for the PAHs, both to predict carcinogenicity, and to determine a mutagenic MOA, because the theory is robust, in predicting formation of a stable carbonium ion derived from a cytochrome P450-generated bay region diol epoxide, and the data base on many PAHs is quite robust.

For nitrosamines, structure activity relationships could also be useful, because many of these are carcinogenic. To determine carcinogenicity of nitrosamines, one could simply ask first, whether there is an alpha carbon that cytochrome P450 could hydroxylate. If so, then this would indicate that cytochrome

P450 can activate this nitrosamine. Second, one could simply make physical chemical calculations of the probability of formation of a carbonium ion from this hydroxylated nitrosamine, that could bind covalently to DNA. This is theoretical. I would defer to a nitrosamine expert here.

12. Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (section 2.4.9) and in humans (Section 2.4.10).

Section 2.4.9 is written very clearly. I was particularly enthusiastic about the discussion on page 28, para. 2, lines 2-4, discussing the importance of evidence of mutagenesis at the site of tumor formation, in the target organ, which is of course highly relevant to the mutagenic MOA of a carcinogen. In the second paragraph, lines 7-8, yes, the micronucleus test is good here for this correlation, but I believe chromosomal aberrations are much more useful, for example, in the case of benzene and acute myelogenous leukemia, multiple myeloma, and many other types of leukemias and lymphomas that benzene induces in humans. Benzene is a very strong clastogen, and a very strong inducer of many types of leukemia, including but not limited to AML: also multiple myeloma and other types of leukemia. I feel that clastogenesis assays are more predictive of benzene-induced leukemia induction than micronucleus tests. There is also a better theoretical reason for this, that clastogenesis can disrupt tumor suppressor genes and cause loss of them from chromosomes, as well as cause translocations, placing a strong promoter next to a previously quiescent, or weakly expressed, cellular proto-oncogene.

On page 28, para. 3, lines 2-3: I recommend changing the way this sentence is written. I suggest the following sentence instead: "Observation of only one mutagenic effect, is suggestive of, but may not conclusively prove, a mutagenic MOA." This is a more positve and precise sentence on this issue.

Section 2.4.10 in general is well-written. In addition to what is already written, it would be helpful to add here on page 29, in para. 1, that induction of mutagenicity in cultured human cells, particularly those from the putative target organ, would also further strengthen the case for a mutagenic MOA for a specific carcinogen. Further, on page 29, the last sentence on this page, running on to page 30 at the top, I recommend changing this sentence and the last sentence of this section. I would recommend a substitution, as follows: "Demonstration of mutations in oncogenes or tumor suppressor gene in tumor tissue is very important data and suggests that mutation may be a necessary and key event in the MOA for carcinogenesis by a specific chemical. Such data definitely adds to the WOE that a mutagenic MOA is operative in human cancer." I would rank such data very highly in assessing whether a chemical carcinogen had a mutagenic mode of action. Otherwise, the authors' writing here is too negative, and they are setting the bar far too high to declare a mutagenic MOA for a specific chemical in the process of carcinogenesis.

13. Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.

Yes, I found Appendix A as a useful way to organize the data from genotoxicity/-mutagenicity studies. Overall, the section is well-written and informative. I would recommend also adding a column to the in vitro studies table, encompassing DNA damage, as measured in the COMET assay and as measured by 8-hydroxy-deoxyguanosine formation in DNA caused by free radical formation. For the in vivo assays, I also recommend adding a row on measurement of 8-hydoxy-deoxyguanosine in DNA to measure DNA damage caused by free radicals.

Regarding Appendix B, I found that this was fairly well-written and clearly written. However, I recommend a sort discussion of the utility of assays to detect morphological and neoplastic cell transformation in cell culture. These assays catch many different types of chemicals, whether they act by

mutagenic mechanisms (BaP), aneuploidization (DES), non-genotoxic/non-mutagenic mechanisms, or by combined mechanisms of action, including genotoxicity (clastogenicty/gene amplification/-micronucleus formation and also enhancement of methylation of tumor suppressor genes (insoluble nickel compounds). These tests have not realized their full carcinogen screening potential. They should be discussed briefly at the end of this section, and a few references added reviewing their utility. Otherwise, this section is fine.

Regarding Appendix C, I found this Appendix appropriately concise and very informative for me and for other readers. I like it the way it is written currently, and I recommend retaining it exactly as it is written. The discussion of the different types of mutagenicity screening regimens accepted by different agencies was very interesting and informative for the reader.

Regarding Appendix D, I like the way this section was written overall. The section on Bone Marrow chromosomal Aberrations/Micronucleus Induction was written very concisely and is very informative in discussing concordance between chromosomal aberrations/micronucleus induction and carcinogenesis by ethylene oxide. The section on Studies on DNA adducts is very informative on the correlation between DNA adduct formation and carcinogenesis by various chemical carcinogens. It is also appropriately concise. The section on the Alkaline Single Cell Electrophoresis Assay (Comet Assay) was also very informative and very concise. I would recommend adding a table to summarize these results, if the authors have the time and the energy, to break up the text and make the results visually more interesting. This is very useful information. The section on In Vivo Transgenic Models is very interesting, informative, contains powerful data, and is appropriately concise. A summary table of this data would also be very interesting, if the authors had the time, energy, and inclination to do this. This data showing a concordance between organ specific carcinogenesis and mutation in that same organ is excellent and very interesting data, and goes a long way toward establishing a mutagenic MOA for the specific chemicals mentioned in the text. The section on Use of Toxicogenomic Data is appropriately concise, informative, and appropriately conservatively written for this novel technology.

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

In addition to answering the Charge Questions, I have written an overall review, including General Comments and Specific Comments, below.

General Comments

This document describes a Framework for Determining a Mutagenic Mode of Action for Carcinogenicity, using EPA's 2005 Cancer Guidelines and Supplemental Guidance for Assessing Susceptibility From Early-Life Exposure to Carcinogens. In general, the document is written in a clear and concise fashion. This document is very useful in that it provides approaches for determining whether a specific chemical has an MOA involved in carcinogenesis. In general, the document is written clearly and concisely and is informative for the reader. The appendices are very useful for providing very informative material on mutagenicity testing schemes at EPA and other places, use of SAR in assessing mutagenicity, and correlation and concordance of DNA adducts, comet results, and mutations in the target tissue, with organ-specific carcinogenesis.

This document should provide a very good framework for determining whether a specific chemical has a mutagenic mode of action or not if its guidelines are followed by risk assessors.

Of course, one should always work to regulate chemical carcinogens based on strong scientific evidence and knowledge of the MOA for cancer, where that exists. However, I am concerned that the tone

of this document is such that it may so raise the bar for deciding that a chemical can act through a mutagenic MOA, that the document can have the undesirable effect of moving much of risk assessment away from the current linear, no threshold default MOA for carcinogenesis for many chemicals. This would result in a weakening of the current conservative carcinogen risk assessment and regulation approach toward regulating carcinogens. I would rather see statements in the document such that, "If a chemical is mutagenic, it is presumed that it has a mutagenic MOA for carcinogenesis, unless strong evidence is presented to the contrary." This would have the effect of protecting public health.

In addition, there is no mention of agents that act through a combination of genotoxic and mutagenic events. A good example of this would be insoluble nickel compounds, which are genotoxic (they cause chromosome damage and gene amplification, and micronucleus formation) and they also cause epigenetic events (methylation of tumor suppressor genes). How are such chemicals to be regulated? My recommendation is that the document state that such compounds would be regulated as having a genotoxic MOA, unless there was evidence to the contrary, or unless there was evidence of a non-mutagenic MOA that was operant at concentrations far below those at which mutagenesis occurred, again to be protective of public health.

Overall, it would have been better in this reviewer's opinion, to entitle this document, "Framework for Determining a Genotoxic Mode of Action for Carcinogenicity." This would have been a more inclusive document, given all the work that is invested in these documents anyway, and would have divided the universe of carcinogens into genotoxic and non-genotoxic. This would have made the application of this Framework to Risk Assessment much more valuable to EPA and the outside community. It would also make more sense, since comet assay results are discussed in here as well. I have made a number of specific comments, and also answered the charge questions, below. When this document is revised along the lines I have suggested, then it could represent a valuable contribution to the U. E. EPA's methodology for conducting risk assessment on mutagenic chemical carcinogens, as a useful adjunct to the two documents, "Guidelines for Carcinogen Risk Assessment," and Supplemental Guidance for Assessing Susceptibility From Early-life Exposure to Carcinogens." I have detailed a number of specific comments, criticisms, and suggestions/recommendations below.

Specific Comments

Page 4, para. 3: This paragraph is very good, because it clearly indicates that the information presented here is not a checklist nor a specific set of criteria that must be met for determining if the WOE supports a mutagenic MOA, but simply provides a framework for organizing data, determining relevance of the data, and considering issues in determining a mutagenic MOA for cancer. This is very appropriate.

Page 8, para. 2, lines 5-6: I recommend changing this statement. It should rather read, "If a chemical is mutagenic, unless there is evidence to the contrary, it is presumed that this chemical acts to induce cancer by a mutagenic MOA. This is a default position."
This would be a reasonable way to proceed, so the risk assessors are not tied up in knots forever trying to decide on an MOA, which might otherwise hold up conducting and completing risk assessments.

Page 8, par. 3, lines 3-5: This should be changed to, "Most genetic toxicologists make a distinction between mutagenicity and genotoxicity, considering that mutagenicity is a subset of genotoxicity. This is commonly accepted practice."

Page 8, para. 4: I disagree with these definitions. This paragraph mixes up "mutagenicity" and "genotoxicity." It is appropriate to define genotoxicty broadly, and to define mutagenicity as a subset of genotoxicty, and to define mutagenicity broadly within its definition, including aneugencity as a subset of mutagenicity. However, I would consider unscheduled DNA synthesis and sister chromatid exchange

under genotoxicity, not under mutagenicity, because there is no strong evidence that they are necessarily mutagenic events, unless the repair is error-prone, although they are clearly genotoxic events. Please tighten up the terminology here.

Page 17, para. 1, gives very short shrift to assays to detect morphological cell transformation. They are very useful, particularly when one does not have 3 years or $3,000,000. to conduct an animal carcinogenesis bioassay. In addition, chemicals can be tested for ability to induce morphological cell transformation, and also for their ability to induce mutation in the same or closely related cell types in vitro. This can give information on whether a chemical has a mutagenic MOA or a non-mutagenic MOA. Here is one example (there are many) of such a paper from the scientific literature, where BaP and N-acetoxy-AFF both induce mutation to ouabain resistance and morphological cell transformation (foci) over the exact same dose ranges, suggesting that these chemicals induce morphological cell transformation, hence likely also carcinogenesis, by a mutagenic MOA::

1. Landolph, J. R. and Heidelberger, C. Chemical carcinogens produce mutations to ouabain resistance in transformable C3H10T1/2 Cl 8 mouse fibroblasts. P. N. A S. USA, 76 (2): 930-934, l979. This paper showed that BaP, and separately N-acetoxy-AAF, induced mutations to ouabain resistance and morphological cell transformation over the same concentration ranges. Hence, this provided evidence for a mutagenic MOA for morphological cell transformation, hence likely also carcinogenesis, for both carcinogens.

Page 31, Section 3.0, lines 3-5: I recommend changing this sentence to, "In the absence of early-life studies on the specific chemicals under consideration, early-life susceptibility is assumed for carcinogens operating through a mutagenic MOA, as well as for those operating through a non-mutagenic MOA."

15. This reviewer's reaction to public comments submitted on this document:

Many of the public comments were very helpful. I am going to cite a few parts of a few responders, only for lack of time, to indicate which are of high priority and should be seriously considered in this document:

a. Regarding the comments from the Natural Resources Defense Council, I agree that non-mutagenic and mutagenic carcinogens are equally dangerous for humans. I also agree that "Increased susceptibility of in utero and early life-stage and sensitive populations should be assumed for both mutagenic and non-mutagenic carciogenic agents." I also agree with the NRDC that the Framework should "clearly state that if a carcinogenic chemical is shown to interact with DNA, it should be presumed to be mutagenic unless demonstrated otherwise…" I refer to this as a default position, and I agree with this presumption. I also agree with NRDC that "health-protective assumption are the default, and must always be the approach used, unless informed otherwise.." This default presumption should be stated explicitly in the document. I also agree with NRCD that, "A mutagenic MOA determination should not require certainty." Otherwise, vested interests could slow down the production and acceptance of a reasonable risk assessment document infinitely. This has already happened with a number of chemicals. I also agree with NRDC that "The Framework must not be overly burdensome for regulators to implement." In addition, I agree that "Early life-stage susceptibility considerations should be extend to all carcinogens."

c. Dr. Butterworth's paper was very interesting. Regarding page 3 of his paper, I agree that the EPA default assumption is, and should remain, that, unless proven otherwise, a carcinogen is considered to be acting through a DNA reactive, mutagenic mode of action. Dr. Butterworth raises the interesting question that even non-genotoxic chemicals occasionally show genotoxicity. He indicates that it is very difficult to

obtain judgement that a chemical acts via a non-genotoxic MOA for this reason. This is a very interesting observation. It complicates the situation for this Framework, and should be considered further Regarding Dr. Butterworth's point #4, I agree that it would be useful to consider some non-genotoxic carcinogens as practical examples to this Framework, and also instructive and informative for risk assessors.

d. Regarding the comments of Dr. Melinck from NIEHS, I agree with Dr. Melnick that placing the requirement for a mutagenic MOA for carcinogenicity, and that mutational activity was a key event in the chemical's carcinogenicity, in order for linear low-dose extrapolation to occur, is a burden that is too stringent for protecting public health. For some chemicals, it may take decades to ascertain whether or not a mutgenic MOA for carcinogenicity occurs. Hence, I recommend the linear, no threshold dose extrapolation currently used as a default be continued for carcinogens until a precise MOA can be ascertained.

e. The comments of the Childrens Health Protection Advisory Committee of OEHHA/California EPA, chaired by Dr. Melanie Marty, were very professional and extensive. I agree with Dr. Marty and the CHPAC that all efforts should be made to ensure that there is protection against childhood exposure to carcinogens. I also agree that as a first principle, it should be assumed "that genotoxic carcinogensi have a mutagenic MOA as the default risk assessment position, and that assessment of risk from such carcinogens warrants application of age-dependent-adjustment factors (ADAFs) to account for early-life susceptibility." These are crucial points that will serve to protect public health, and they should be incorporated into this Framework. I agree with the CHPAC that there should be no further restriction of the application of the ADAFs. CHPAC comments that ADAFs could be applied only if additional data beyond standard genetic toxicology tests are available, and that such data are often not available or highly uncertain, is important. I agree. I also recommend applying ADAFs to children regardless of the mechanism of carcinogencity, to be protective of public health, particularly that of children. I also agree with CHPAC's comments 5) on page 3, that the deinfition of mutagen in the remwwork is too limiting. As indicated in my independent review, I recommend broadening the definition of mutagen to include clastogens, aneugens, and oxygen radical-generating agents. Further study should be made of CHPAC's assertion in point 6), that, "The Framework would likely fail when tested against some of the 12 mutagenic carcinogens upon which the Supplemental Guidance is based." I recommend an exercise conducted between CHPAC/OEHHA and U. S. EPA to test this contention, before the Guidelines are finalized.

I also agree with CHPAC that U. S. EPA should revise the Framework such that genotoxic carcinogens are assumed to have a mutagenic MOAs unless proven otherwise. This is exactly my own independent position on the Guidelines, and is a position that is protective of public health. I also agree that the "Framework should use an inclusive default approach that considers both direct-and indirect-acting mutagens and genotoxic carcinogens as possessing a mutagenic MOA that warrants application of the ADAFs for early–life susceptibility." I strongly recommend, and I would be very pleased to see, more cooperation between OEHHA/Cal. EPA and the U. S. EPA, to work to make these Guidelines as strong and appropriate as possible to protect public health.

f. Regarding extensive comments from the American Chemistry Council (ACC), I agree with their comment on page 7 that, "There is no guidance on the interpretation of data on DNA modification induced by reactive oxygen species." I have independently made a similar comment, and the Framework as revised should reflect this. In addition, on page 7, I also agree that DNA strand breakage should be referred to as a genotoxic event, not a mutagenic event.

g. Regarding the very extensive and very comprehensive comments form Dr. Richard J. Albertini and Vernon E Walker, I completely agree, again, that the Framework should distinguish between "genotoxic," as the higher, more inclusive level term, and "mutgenic" as a subset of "genotoxic," and that indirect

mutagens should also be considered in this Framework. These authors' definitions of genotoxic and mutagenic on page 4, para. 1, are very precise. I agree with them, and recommend that EPA incorporate these definitions into the Framework. I also agree with these authors' discussions on page 6 and page 7 carefully distinguishing between genotoxic events and mutations. I recommend that EPA incorporate these into the Framework also. On page 15 and 16, these authors' recommendations are good ones and I agree with them. I have made many similar recommendations in my own independent review. Certainly, these authors are correct in distinguishing between pro-mutagenic and no-pro-mutagenic adducts, and in pointing out that DNA strand breaks, UDS, and SCE do not per se constitute evidence of mutations and should not be equated with mutations. I have made similar points in my own review. I recommend that these points be incorporated into the Framework. I also agree and have pointed out in my review that direct DNA reactive mutagenicity and indirect mutagenicity should be distinguished in the Framework. These authors' review is excellent, and I cannot give justice to it in this short space. I strongly recommend that EPA consider the points made in this paper very carefully, and try to incorporate as many of them as possible into the Framework.

h. I have read the comments of Dr. George Alexeeff, Deputy Driector for Scientific Affairs, OEHHA/California E. P. A. with interest. I agree with Dr. Alexeeff that the premise of this Framework, that a carcinogen does not have a mutagenic MOA until one can provide enough information to state with a high degree of certainty that it does, conflicts with the assumption of utilizing linear low-dose exptrapolation as the long-standing default position for carcinogen risk assessment. I recommend that this issue be dealt with upfront in the Framework. I also agree with Dr. Alexeef that the Freamwork should make it clear that there may not be only one primary, MOA, applicable across life-stages. He also makes the interesting point that the MOA may not be the same across doses, which is a very important point, and complicates use of one MOA for one chemical. He also makes important points in his section 7, Mutations in cancer-relevant genes, and 8. Weight of Evidence for a Mutagenic MOA, that should be addressed in the Framework. Again, I agree independently with his position that the default position for risk assessment should still prevail. His suggestion of pilot testing this Framework with test carcinogens is a very good one. His point that many chemical carcinogens act by multiple modes of action is important, and one I have also discussed in my own independent review. I very much agree with his contention that "These uncertainties point toward a default assumption that carcinogens act via a mutagenic mode of action unless data to the contrary exist for specific carcinogens." I would recommend that EPA pay serious attention to the comments of Dr. Alexeef, a very sophisticated and experienced risk assessment professional.

Bette Meek

RESPONSES TO THE TECHNICAL CHARGE TO PEER REVIEWERS

External Peer Review of U.S. EPA's draft *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity (Framework)*

Task Order No. 9
Contract No. EP-C-07-024
February 20, 2008

PRE-MEETING COMMENTS ARE DUE TO ERG BY CLOSE OF BUSINESS, WEDNESDAY, MARCH 26, 2008

CHARGE QUESTIONS

1. The purpose of the *Framework* is to provide an overall process by which chemicals can be evaluated and a determination made regarding whether the chemical has a mutagenic mode of action (MOA) for carcinogenicity. MOA is described as the key decision in risk assessment in U.S. EPA's 2005 *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*, and the accompanying *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (Supplemental Guidance)* highlights the importance of ascertaining whether a chemical has a mutagenic MOA. The *Framework* document recognizes both the on-going research in this area, as well as the potential limitations of available data for making this determination.
   a. Please comment on whether the purpose for the document is clear.
   b. Does the document provide a useful framework for this determination?
   c. Is the document clear, complete and objective?
   d. Are major limitations and assumptions clearly defined?

Response:

Firstly, continuing emphasis of the EPA in formally and transparently considering modes of action as a basis to inform relevance of observed effects to humans and meaningful characterization of interspecies differences and human variability in subsequent dose-response analysis is laudable and fully supported by this reviewer. Comments below are offered principally in the context of the desirability of providing guidance to fully reflect the current considerable experience in formal consideration of the weight of evidence for modes of action for cancer and meaningfully sharing this experience with assessors in the agency. .

In relation to the points raised in this question, while the purpose of the document seems clear, it appears not to be sufficiently well developed from the perspective of robustly considering mode of action for

substances for which a critical early key event in induction of cancer is direct interaction with DNA. In fact, it appears to reflect the perhaps greater experience of the authors in considering weight of evidence for hazard (in this mutagenicity) in a screening context based on the results of batteries of *in vitro* and *in vivo* assays for genotoxicity. This must necessarily be distinguished from the more appropriate consideration of mutagenicity data in the context of the weight of evidence of modes of action for cancer which include a critical mutagenic component.

On this basis, I believe that the document would benefit from complete restructuring to consider initially, the mode of action framework for induction of specific tumours. The appropriate information on mutagenicity/genotoxicity would then be considered in the context of the hypothesized mode of induction of tumours, rather than being based on the largely accepted principles for considering weight of evidence for interaction with DNA in a screening context. This will necessarily result in emphasis on specifically relevant information such as mutation in the critical tissue for the tumour of interest.

Emphasis on the mode of action determination taking into account the critical key events, some of which may include direct or indirect interaction with DNA also permits informed characterization of dose-response, depending upon which key events are rate limiting.

So while the document contributes to the important objective of formal consideration of mode of action to meaningfully inform estimation of risk, in its current form, it doesn't appear to reflect considerable experience for incorporation of relevant information on interaction with DNA as a critical key event in a hypothesized mode of action for tumours. Its additional development in this context could be facilitated through inclusion of case studies for subsequent consideration in (a) workshop(s) of experts, including both genetic toxicologists and those with experience in conducting weight of evidence determinations for mode of induction of cancer. This is suggested as one potential option in integrating more meaningfully, relevant information on nature of interaction with DNA as a critical key event in the mode of action for specific tumours.

The current emphasis of Section 2.3, for example, is consideration of the weight of evidence for interaction with DNA based on assays developed principally for hazard identification in a screening context. This is presented distinctly from the content of section 2.4, which includes reference to criteria in frameworks to consider MOA but with limited reference to the types of information that are most relevant in considering mutagenicity as a critical early key event in specific hypothesized modes of action for induction of specific tumours.

In assessing potential modes of action for carcinogenicity, key events leading to specific observed tumours are considered. These can include direct and indirect interaction with DNA. This consideration of potential key events in the tissue of interest frames the nature of data on interaction with DNA that should be weighted, with emphasis on tissue specific information relevant not only to the weight of evidence for mode of action but also to subsequent implications for dose-response. In this context, I find myself questioning the relevance of the lengthy discussion in Section 2.3.2 regarding "categorizing data" and "describing the WOE for mutagenicity".

Individual results must necessarily be considered, then, in the context of the hypothesized mode of action for specific tumours. Characterizing the weight of evidence of all genotoxicity or mutagenicity data on the basis predominantly of screening assays for hazard doesn't really contribute to this objective. My strong preference, then, is to delete the current sections 2.1 to 2.3 and rewrite the text included therein to be fully integrated within a mode of action context based on the types of considerations that are made for specific tumours.

In relation to clarity of the document, there is occasionally confusing and sometimes seemingly contradictory reference to effects considered to represent "mutation". This arises, in part, as a consequence of the operational definition of "mutagenicity" within the somewhat narrow context of a mutagenic mode of action for carcinogenicity (see detailed comments, for example, from Albertini and Walker). The content could also be considered to be somewhat misleading, currently, in relation to the appropriate consideration of the weight of evidence for mutagenicity based on results of screening assays for hazard in the context of a mode of action analysis for cancer.

As a basis for providing meaningful guidance in a complex area, it would be extremely helpful to include some case studies where a mode of action analysis is conducted for a substance or substances where direct and/or indirect interaction with DNA are likely critical early key events in the induction of specific tumours. I believe that these case studies would be extremely important in demonstrating the need to more meaningfully consider available data on the mutagenicity of substances in the context of an hypothesized mode of action for specific tumours – i.e., to shift the emphasis on consideration of weight of evidence for hazard (i.e., mutagenicity) based on screening assays to consideration of and identification of critical data gaps in relation to more relevant information in a mode of action context for tumours. These need not be real chemicals but could indeed be based on manufactured data sets, simply to provide the necessary illustrative application. Particularly helpful would be an example for substances

with potentially multiple modes of action where dose-response for rate limiting key events are taken into consideration in the subsequent dose-response analysis. Substances that provide interesting datasets as a starting point for development of such case studies that come to mind include propylene oxide and formaldehyde.

Without additional integration of these components (i.e., interaction with DNA and mode of induction of tumours) which are currently seemingly distinctly considered to address principally mode of action, taking into account relevant information on nature of interaction with DNA with illustration by case studies and/or examples, it is difficult to envisage how the document will meaningfully contribute to consistency across the agency. Also, in addition to providing illustrative application for purposes of increasing understanding and consistency, development of case studies is also extremely helpful in providing feedback to meaningfully revise the framework from both within and outside of the agency.

In relation to the point raised in the question regarding clear definition of major limitations and assumptions, again, while assumptions are largely stated, there are a number of seeming inconsistencies throughout the text in relation to some of those that are critical (e.g., the narrow definition of mutagenicity in the context of this document). Also, a seemingly critical limitation that is not overtly stated is the limited contribution of the types of testing protocols that have been developed to screen mutagenic hazard to robustly address mode of action for tumours – e.g., to meaningfully consider dose-response for potential key mutagenic key events for cancer in specific tissues. Better integration and application of the framework to address these critical datagaps may hopefully result in the generation of data that can inform to a much greater extent in the context of mode of action.

2. Given the inconsistency in the scientific community of genetic toxicologists regarding the definition of mutagenicity, the *Framework* (section 1.4) proposes an *operational* definition of "mutagenicity" for a very limited use, i.e., determination of a mutagenic MOA *for carcinogenicity* under the U.S. EPA *Cancer Guidelines* and *Supplemental Guidance*. Please discuss whether this definition is useful and appropriate, considering the limited application in this *Framework*.

Operational restriction of the term "mutagenic" in the framework as the capacity of either the carcinogen or its metabolite to react with or bind to DNA in a manner that causes mutation facilitates reliance on the results of standard testing protocols for hazard identification, given that mutagens in this context usually (though not always) produce positive effects in multiple standard test systems for different genetic endpoints. However, there are clearly other forms of interaction with DNA which are likely key events in the induction of cancer and their role needs to be carefully considered in a mode of action context. The basis for restriction then, of the definition in a mode of action context is somewhat unclear. Again, this

would seem to be a function of current limited application of mode of action frameworks to encourage development of appropriate *in vivo* data that will inform the dose-response for limiting key events, which may include both mutagenic (as defined here) and non mutagenic interaction with DNA.

3. Section 1.2 (uses of mutagenicity data) was added in coordination with and at the request of other Federal agencies that wished to distinguish their use of such data from the use in this *Framework*.
   a. Is this section clear and sufficiently complete to accomplish that goal? Please suggest recommendations to improve this section.
   b. If this section is not complete, what other specific uses of mutagenicity data for regulatory purposes might be addressed?

I think that inclusion of this section offers considerable opportunity to clarify that data collected for hazard identification purposes in a screening context are not really designed to meaningfully assess MOA for specific tumours. In fact, I believe it is somewhat misleading to indicate that these screening protocols for hazard "can provide high quality data useful in assessing MOA". I'd suggest that it be overtly stated that these types of data are collected generally for a completely different purpose and may or may not be relevant in consideration of the hypothesized mode of induction of a specific tumour.

Potentially considerably more informative data are those on dose-response for key events (including interaction with DNA) *in vivo*, in the target tissue of interest (i.e., those in which tumours occur). Note for example, the potential inconsistency of the statement quoted above with information on weighting of relevant data in Section 2.4.9 on support for the Mutagenic MOA for Carcinogenesis in Animals which states: "For establishing a mutagenic MOA, *in vivo* data are most useful when genetic damage is examined in the target organ." So, while the screening genetic toxicity assays are appropriate for the purpose for which they were designed (i.e., screening of hazard often to determine additional testing requirements), it is more investigation of the nature of the dose-response curve for early critical key events including mutagenicity in the target tissue which will necessarily inform mode of action analyses for cancer. It seems important that the text of the document consistently reflects this understanding.

4. Which version of Figure 1 best captures the steps proposed in the *Framework*? Please identify the basis for your recommendation.

I would suggest revision of Figure 1 to better reflect the state of development and application of mode of action analyses for cancer, taking into account early, critical key events including direct interaction with DNA. As per comments above, available data on genetic toxicity should be weighted in the context of their contribution to an hypothesized mode of action for particular tumours in a target tissue. Since weight of evidence determinations based on screening assays for hazard (mutagenicity) have limited application

in this context, in my view, then, the figure would indicate the specific tumour of interest, then apply the cancer guidelines MOA framework based on the hypothesized mode of action, weighting the most relevant (tissue-specific) mutagenicity data (as indicated in the list on page 2 of Figure 1, version 2). To the extent possible, dose-response for rate limiting key events including those that involve direct interaction with DNA would inform subsequent extrapolations. Other mutagenicity data would be considered in a supporting context, principally from the perspective of determining whether or not the pattern of results observed across the different screening assays is what might have been expected, based on the hypothesized mode of action. This analysis would necessarily need to be repeated for each different tumour type, unless modes of action were similar.

5. The MOA framework is defined in the *Cancer Guidelines.* The steps are repeated in the *Framework* with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.
   a. Is the information in this *Framework* on the application of the *Cancer Guidelines* MOA framework sufficiently clear and complete to be useful?
   b. Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data that are generally available for consideration? If not, what additional elements might be useful to include in the *Framework*?
   c. Please review each step, e.g., "key events," "dose-response relationships," "temporality," and discuss what types of additional information might be added (remembering that this document can not suggest or require additional testing). Given the current state of science, please comment on the application of each step in the MOA framework.

I think that the MOA framework has been faithfully reproduced in the current document including discussion as it applies to determining a mutagenic MOA for carcinogenicity. However, guidance could be much clearer, if the text was illustrated by case studies including chemicals with multiple modes of action for carcinogenicity involving both direct and indirect interaction with DNA.

Also, as per previous comments, I would suggest restructuring this section. Rather than beginning with a weight of evidence determination of hazard that a chemical or metabolite is mutagenic, it is rather more important that the weight of evidence for an hypothesized mode of induction of specific tumours be considered, taking into account relevant data on interaction with DNA . The most relevant data in this context will generally be those relevant to the specific tissue, which inform dose-response analyses for limiting key events.

It would also be important to emphasize at the outset, that each tumour type requires a separate analysis with the exception of those that are induced by the same hypothesized MOA (This currently doesn't appear until well through the section, as a result of the considerable emphasis at the outset of the section

on weight of evidence for mutagenicity based on screening assays). This more appropriately sets the scene for considering relevant data on interaction with DNA in the context of its relevance to the hypothesized mode of action. Again, in my view, this would be best demonstrated by example, with levels of complexity similar to those commonly encountered in conducting risk assessments (i.e., including multiple modes of action).

For example, if the hypothesized mode of induction of a particular tumour type involves GSH depletion which induces cell proliferation and loss of protection against endogenously generated reactive oxygen species and/or the enhancement and manifestation of direct DNA-reactive mutagenicity of weak mutagens by inhibiting their detoxification in the presence of increased cell proliferation, how would consideration of the weight of evidence of mutagenicity as described in Section 2.3 meaningfully inform this hypothesized mode of action?

Also, by way of example, lines 23 to 37 on page 20 indicate that for chemicals considered to have a mutagenic MOA, results will generally include positive findings in one or more *in vivo* studies that are generally supported by *in vitro* gene mutation or cytogenetic assays. The text goes on to state (lines 33 to 35) that one would generally expect positive results in more than one organ or tissue as well as positive *in vivo* test results from more than one phylogenetically distinct species. However, again, there is no attempt to relate this information to hypothesized modes of action for specific tumours, even by way of example. This rather espouses commonly accepted principles for consideration of weight of evidence for hazard for interaction with DNA in a screening context but seemingly falls short of meeting the intended objective in this document of more robustly considering the nature of this information relevant to consideration of tissue specific modes of induction of cancer.

Even in the section which addresses the MOA framework, reference is principally to weight of evidence hazard determinations for mutagenicity based principally on screening assays, for which no context to envisaged potential modes of action is provided. Maintenance of the "status quo" in this context (i.e., continued reliance on these testing strategies designed for hazard identification principally in a screening context) will almost certainly ensure that limited progress is made in elucidating modes of action for induction of cancer including mutagenic key events.

It should be noted that one of the significant advantages of consideration of relevant data in mode of action/human relevance frameworks in addition to their permitting transparent and informed organization of data as a basis to consider weight of evidence is their objective to clearly delineate critical data gaps.

The considerable evolving experience in application of these frameworks has also advanced consideration of the more generic nature of relevant data on mutagenicity which can best inform mode of action analyses for cancer. Additional reflection of this experience within the document would be beneficial.

There is also text on page 21 concerning consistency of effects across different assays, induction of more than one type of genetic effect and observation of effects *in vivo* versus *in vitro*. Again, this will depend very much on the hypothesized mode of induction of specific tumours and how or whether the particular assay addresses envisaged key events. For this reason, it is agreed that as mentioned in the middle of page 21, *in vitro* results are not automatically overruled by negative *in vivo* results. This is necessarily a function of relevance to the hypothesized mode of action of the *in vitro* study and the relevance and nature of the testing conducted *in vivo (*i.e., the tissue of interest at dose levels of interest?).

Also in relation to the reference to the potential value of SAR on page 22, in my view, the value of this information is not really restricted to increasing the weight of evidence consistent with data from mutagenicity testing. Given that this statement appears in the section on mode of action, I'd proposed that SAR is extremely helpful in considering the weight of evidence for modes of induction of particular tumours, where there is an established mode of action for similar types of tumours (including mutagenic key events) for structurally related chemicals. This is yet another example of the inappropriate focus (in my view) on weight of evidence for mutagenicity, based on consideration of the results of principally screening assays for hazard, without context in relation to hypothesized modes of action for tumours.

Further in Section 2.4.1 – at the end of the first paragraph, it is stated that "For a mutagenic MOA, mutation is the first step which initiates a cascade of other key events such as cytotoxicity or cell proliferation that are key to the carcinogenic process. This is a relatively stringent criterion and in reality, there may be multiple contributing modes where mutation is but one component and not necessarily the first one. Rather, what is critical to inform the subsequent dose-response analysis is an understanding of the key events that are limiting for any specific tumours. The statement above could be interpreted to be somewhat contradictory to one that appears three paragraphs later where it is indicated that "The critical question posed at this stage in the evaluation is this: "Is mutation an early key event in this chemical's induction of cancer" (rather than the "first" event). Interestingly, while seemingly pivotal, there is almost no guidance provided in this document as to how it might be determined that mutation is an early event in carcinogenesis in a mode of action context.

In relation to the criteria listed at the bottom of page 23, its somewhat difficult to understand how criteria such as "tumors are observed in multiple sites, in multiple species and from multiple routes of exposure" can be weighted in the current framework to consider mode of action for individual tumour types. In my view, this should probably relate to whether or not there are more than one type of tumours induced by the hypothesized mode of action? The same is true for the criteria indicated as "tumor responses generally occur early in chronic studies". In fact, these observations are not very informative really from the perspective of elucidating key events in the hypothesized mode of action for specific tumours. Inclusion of these criteria seems to relate to weight of evidence for hazard versus elucidation of mode of action and consideration of weight of evidence for key events in animals and humans.

In Section 2.4.4 ("Dose-Response Relationships"), it is probably important to indicate that early key events are expected to be observed at doses below or similar to those where tumours are observed. In addition, the incidence of early key events is expected to be greater to or equal than that for the end event.

For Section 2.4.5, given the rather stringent criterion that mutation be the first or at least an early step, it would seem important to provide an example of the type of data that can inform this determination. Currently, the only example provided in the paragraph relates to another criterion, namely that for dose-response (i.e., "To the extent that the mutagenic events occur earlier than or at lower doses than the tumours……..")

For biological plausibility and coherence, another example of relevant data/observations is whether or not the pattern of effects across species/strains is consistent with the hypothesized mode of action (e.g., metabolism to the active entity). As appropriately pointed out, it can also include consideration of information from structurally related chemicals where mode of action has been considered.

For Section 2.4.7, its not so much just that modes of action act simultaneously but that more than one may contribute to the observed effect. Rather than analyzing them independently, then, what is desirable is to integrate and define to the extent possible, dose-response for both mutagenic and non mutagenic key events in an hypothesized mode of action to determine which are rate limiting.

For Section 2.4.8, focus of the discussion on inconsistencies relates principally to results of screening assays for mutagenicity, rather than their context in relation to an hypothesized mode of action. A critically important objective of frameworks for considering the weight of evidence for hypothesized modes of action and their human relevance relates to delineation of additional critical data that would be

helpful to"make the case". Based on evolving experience in this area, it is unlikely that these will be the kinds of screening assays for mutagenicity conducted principally to determine the need for further testing. Rather, they will likely be determination of a marker in the relevant tissue (i.e., where tumours occur) at relevant dose levels.

For Section 2.4.9, in relation to the last paragraph, again, this necessarily depends on the nature of the data which demonstrates that the substance is a systemic mutagen to the hypothesized mode of induction of the specific tumour of interest.

In relation to Section 2.4.10, in fact epidemiological data, particularly that collected with no view to informing whether or not a particular mode of action is operating in humans (i.e., incorporating one or more biological markers of key events) are not all that helpful in "simplifying" the analysis, particularly where the results are negative. Information on mode of induction of tumours in animals can be helpful though, in interpreting epidemiological data (e.g., whether tumour sites are likely to be concordant).

In addition to the types of information outlined here as a basis to "inform" consideration of human relevance (i.e., chemical-related toxicokinetic and toxicodynamic data), it is important to consider non-chemical related factors such as physiological, anatomical and biochemical variations between animals and human which could impact either qualitatively or quantitatively on human relevance.

Concordance tables which clearly indicate the extent of chemical-specific evidence available for key events in animals and humans and consideration of a broader range of factors which impact on human relevance (i.e., as a basis to characterize both qualitative and quantitative toxicokinetic and toxicodynamic variations between animals and humans) are extremely helpful in increasing transparency in interpreting available data (See, for example, Meek et al., 2003; Seed et al., 2005; Boobis et al., 2006; Boobis et al., 2008). These factors include the nature of the appropriate metabolic pathway in humans, anatomical differences which could lead to different outcome for effects associated with physical interaction with tissues and human disease states relevant to interpretation of the hypothesized mode of action. It is also extremely helpful to additionally frame consideration of human relevance in the context of both potentially qualitative and quantitative differences. While hypothesized modes of action for observed tumours in animals are rarely qualitatively irrelevant to humans, the quantitative data on key events considered in the analysis of human relevance are critically important to inform subsequent dose-response characterization (i.e., for those key events which are limiting).

6. Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader? Does it adequately describe how the quality of the data can be evaluated? If not, how could it be expanded or improved? Please provide specific recommendations.

For the discussion of evaluation of the data against current acceptance and quality criteria, it is important to recognize that studies most suited to consideration of the weight of evidence for a mode of action including mutagenic components are not necessarily those defined in standard testing protocols developed principally for screening of hazard. Indeed, consistent with the content of other parts of the document, this section seems to address the acceptability of data generated in screening genotox assays designed not as a basis to investigate hypothesized modes of induction of specific tumours, but rather, principally as a basis to consider the need for additional testing. Revision of the document to better reflect the status of experience concerning the nature of information that is likely to best inform mutagenic modes of action analyses for cancer would impact significantly on the content of this section.

7. Are the discussions of aneuploidy adequate? If not, how should they be expanded? Please provide specific examples and detailed information regarding recommendations.

I found only one reference to aneuploidy (on the bottom of page 17) which indicates that aneuploidy is a common occurrence in certain tumour types but such changes in chromosome number may not occur through the same mechanisms that produce "other" mutations. The text goes on to state further that positive results in this assay occur for some chemicals that are negative in multiple tests for gene mutations, chromosome mutations and DNA effects and as a result, positive results only in this assay are less likely to support a WOE determination for a mutagenic MOA for carcinogenesis. While the text here helpfully makes the distinction that the implications of positive results of these types of assays need to be carefully considered taking into account other data, again this is presented more in the context of weight of evidence for the results of screening tests for genotoxicity rather data more relevant in a mode of action context. What might be more helpful here is provision of an example of the contribution of such information in a specific hypothesized mode of action for tumour development.

8. Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutational event, or knowledge of mutational spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.

This would seem to be the type of information that is critically important in the context of weight of evidence for a hypothesized mode of action as a basis for interpretation that the mutational event is relevant to the tissue in which tumours are observed rather than considering the overall weight of evidence for hazard based on assays developed principally for screening. It is also pretty much essential, in my view, to provide rationale as to how and why, the pattern of effects observed in (principally)

screening assays for mutagenicity (hazard) are relevant to the mode of induction of the observed tumours being considered in a mode of action analysis.

9. Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.

In my view, it is always important to consider all relevant data in a mode of action analyses for observed tumours, though the extent of this analysis is necessarily dependent upon the availability of relevant data. Moreover, in relation to priorities, it is most critical for substances where margins between estimated exposure and doses associated with effect are relatively small.

Even where there are limited data, where tumours have been observed, structured and thoughtful consideration of available information in a mode of action/human relevance context may bring to light additional (often non chemical specific) considerations relevant to subsequent steps in the risk assessment. It also facilitates discussion among and increases the transparency of guidance provided by risk assessors/regulatory agencies to researchers/stakeholders regarding the specific nature of additional data that would meaningfully "inform" the understanding of mode of action. Increased transparency on this front is essential to meaningful investment of public and private funds to focus on critical research needs rather than "data gaps".

Specific reference in this paragraph to data on structurally related chemicals and formation of reactive species in this context seems appropriate. One can well imagine other relevant situations, where such an analyses might be advised, such as data on mutation in the target tissue in the absence of weight of evidence of mutagenicity based on the results of principally screening assays.

10. The WOE for determining an MOA for carcinogenicity is described and determined in the U.S. EPA *Cancer Guidelines*. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss if we have achieved this goal. If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?

This question addresses aspects seemingly very similar to that referenced in #5. For the reasons mentioned in my response to this and other questions, I believe that the draft document has only partially met the goal of organizing the data in a relevant context, determining the relevance of those data and considering issues in a mutagenic MOA. This seems to be a function of its drawing to only a limited extent on rapidly evolving experience in application of mode of action/human relevance frameworks. As

a result, there seems to be undue emphasis on considering "weight of evidence" for mutagenicity based on assessment of *in vitro* and *in vivo* screening assays for hazard.

As per more specific comments indicated in response to question 5, greater emphasis and more consistent reference throughout the text to the nature of data which informs mode of action analyses for cancer is critical. This includes data on dose response for key events in the tissue of interest including mutagenicity. Subsequent human relevance analysis which addresses qualitatively and quantitatively toxicokinetic and toxicodynamic differences, drawing transparently and broadly on chemical specific information and that on comparative physiology, anatomy, biochemistry, etc. is also key. It would also be helpful to include additional examples within the current text as a basis to illustrate concepts and to develop case studies as a basis to meaningfully inform application of the guidance.

Suggested revisions to the framework and text in this document describing same include more explicit consideration of relevant aspects in relation to determination of whether or not the weight of evidence for the hypothesized mode of action fulfils the Bradford Hill criteria such as an indication for dose-response that early key events are expected to be observed at doses below or similar to those where tumours are observed and that incidence is expected to be greater to or equal than the end event (in this case, tumours). Given the rather stringent criterion that mutation be the first or at least an early step, it would seem important to provide an example of the type of data that can inform this determination. For biological plausibility and coherence, another example of relevant data/observations is whether or not the pattern of effects across species/strains is consistent with the hypothesized mode of action (e.g., metabolism to the active entity). In relation to consideration of other modes of action, it is also important to point out that more than one may contribute to the observed effect and to integrate and define to the extent possible, dose-response for both mutagenic and non mutagenic key events in an hypothesized mode of action to determine those which are rate limiting.

It should additionally be emphasized that a critically important objective of frameworks for considering weight of evidence for hypothesized modes of action and their human relevance relates to the need to delineate clearly additional critical data that would be helpful to"make the case". It should be overtly recognized that it is unlikely that these will be the kinds of screening assays for mutagenicity conducted principally to determine the need for further testing and that the criteria for consideration of weight of evidence for this type of information have limited application in the context of considering mode of action for specific cancers.

In considering human relevance, there should be greater emphasis on consideration of anatomical, physiological, biochemical variations, etc. between animals and humans as a basis for determination of the likelihood of occurrence of key events. Concordance tables which clearly indicate the extent of chemical-specific evidence available for key events in animals and humans and expected relevance based on consideration of a broader range of factors such as the nature of the appropriate metabolic pathway in humans, anatomical differences which could lead to different outcome for effects associated with physical interaction with tissues, and human disease states relevant to interpretation of the hypothesized mode of action can be extremely helpful in increasing transparency in the presentation and interpretation of available data (See, for example, Meek et al., 2003; Seed et al., 2005; Boobis et al., 2006; Boobis et al., 2008). Consideration of human relevance in the context of qualitative and quantitative kinetic and dynamic differences also aids in increasing transparency and more robustly informing the subsequent dose-response analysis.

As a basis for providing meaningful guidance, it would also be extremely helpful to include some case studies where a mode of action analysis is conducted for a substance or substances where direct and/or indirect interaction with DNA are likely critical key events in the induction of specific tumours. I believe that these case studies would be extremely important in demonstrating the need to more meaningfully consider available data on the genotoxicity of substances in the context of an hypothesized mode of action for specific tumours – i.e., to shift the emphasis on weight of evidence for hazard based on screening assays for interaction with DNA to consideration of available and generation of more relevant information in a mode of action context for tumours.

11. For groups of similar compounds, e.g., nitrosoamines or polycyclic aromatic hydrocarbons (PAHs), could a mutagenic MOA be supported solely by structure activity relationships or analogy? Apart from the consideration of groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by structure activity relationships or analogy? If not, what additional information would you think might be needed?

Interpretation of data on structurally similar compounds is necessarily based on weight of evidence considerations, taking into account a number of factors. Several of these have been delineated in a formal approach to consideration of the preliminary weight of evidence for cancer and genotoxicity based on consideration of both data and structure activity modeling/analogy as a basis to contribute to identification of priorities for assessment from among the 23, 000 substances on the Domestic Substances List in Canada.

In this approach, preliminary weight of evidence determinations are based on hierarchical consideration of the results of available studies, QSAR/SAR model predictions, and finally, analogy. Considerations in weighting include consistency across data and/or modelling output, taking into account predictive power of the relevant or underlying bioassays, and sensitivity and specificity of the models, for similar compounds in the training set.

Its important, then, to consider the weight of evidence (i.e., strength, consistency, specificity, biological plausibility, etc.), in relation to the database for structure activity relationships or analogy in making decisions concerning the likelihood of an hypothesized mode of action for tumours which includes an early and predominant role for mutagenicity. However, based on our experience in considering the output of (Q)SAR and analogy, in making informed judgments based on fulsome understanding of the strengths and limitations of various models in the context of their relevance to the compound being considered I'd envisage having sufficient confidence to conclude that mutagenicity may have an early and predominant role in induction of tumours for related compounds.

12. Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (Section 2.4.9) and in humans (Section 2.4.10).

This is addressed also in response to questions 5 and 10, since in my view, assessment of the weight of evidence for the hypothesized mode of action in animals and its human relevance are integral components of frameworks to consider mode of action relevant to risk assessment.

For the human relevance component, there should be greater emphasis on consideration of non-chemical specific information such as anatomical, physiological, biochemical variations, etc. between animals and humans as a basis for determination of the likelihood of occurrence of key events. Concordance tables which indicate the extent of chemical-specific evidence available for key events in animals and humans and expected relevance based on consideration of a broader range of non chemical specific factors such as the nature of the appropriate metabolic pathway in humans, anatomical differences which could lead to different outcome for effects associated with physical interaction with tissues and information on human disease states which inform the nature of toxicokinetic and toxicodynamic variations between animals and humans can be extremely helpful in increasing transparency in the presentation and interpretation of available data (See, for example, Meek et al., 2003; Seed et al., 2005; Boobis et al., 2006; Boobis et al., 2008). Consideration of human relevance explicitly in the context of potential qualitative and quantitative

differences also aids in increasing transparency and more robustly informing the subsequent dose-response analysis.

As a basis for providing meaningful guidance, inclusion of case studies where qualitative and quantitative relevance to humans is considered for a substance or substances where direct and/or indirect interaction with DNA are likely critical key events in the induction of specific tumours would be additionally informative. They would be instrumental in illustrating the rather limited application of genetic toxicology screening assays in this context versus more relevant tissue specific data for the tumour of interest (e.g., text in Section 2.4.9 which indicates "It is important to note that the currently accepted whole animal genetic toxicology assays are not designed to detect the specific mutation(s) that initiate the carcinogenic process……It should be noted that mutations obtained using the target organ or tissue may be used to address the issues of dose response and site concordance and possibly temporal associations.")

13. Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.

I believe that the content of most of the Appendices has limited relevance to the objectives of the guidance, which relates to consideration of mode of action for cancer, taking into account relevant data on mutagenicity. For example, the envisaged value of provision of a template for organization of information concerning the weight of hazard from genotoxicity screening assays is unclear. Presumably, the guidance in this document should relate to interpretation of relevant information on mode of action for cancer where there is a mutagenic component with illustrative examples, rather than identification of format for presentation of screening genetic toxicity results. Specifically, available information will necessarily be weighted in the context of their relevance to an hypothesized mode of action for cancer in a specific tissue and depending on this context and the amount of information available, the results of some and/or many of these assays may have limited relevance. As appropriately indicated at the outset of the table in Appendix A, "The assays included, as well as the organization thereof, depend on the quality and quantity of assay *(stet)* available." I suspect that it would be additionally important to add reference to context in relation to hypothesized mode of induction; these qualifiers might lead one to conclude that the table is not very helpful to the objective of the document and could be deleted.

Similarly, the relevance of inclusion of information on testing schemes and/or use of SAR designed for a completely different objective (i.e., screening of hazard) (Appendices B and C) and selective citation of concordance between results of some of these assays and cancer outcomes (Appendix D) have seemingly

limited relevance to the objectives of the guidance. Wouldn't the more important aspect of interpretation in a mode of action context critical to the task at hand be best illustrated by inclusion of case studies?

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

In summary, the document represents an important step forward in emphasizing the importance of systematically considering mode of action, in interpreting evidence of hazard for application in risk assessment. However, I believe that it could be significantly improved by drawing additionally on the considerable, evolving experience in conducting formal mode of action analyses for both cancer and non-cancer effects to better integrate the seemingly disparate consideration, currently, of the weight of evidence for interaction with DNA based on screening assays versus consideration of mutagenicity as a critical key event in the induction of specific tumours. In this context, inclusion of well developed representative examples of the consideration of the weight of evidence for mutagenic modes of action for specific tumours, would likely additionally inform the intended audience.

References:

Meek, et al. (2003). Crit Revs Toxicol 33:591

Seed et al. (2005) Crit Revs Toxicol 35: 663

Boobis et al. (2006) Crit Revs Toxicol 36:781

Boobis et al. (2008) Crit Revs Toxicol 38:87

Jerry Rice

TECHNICAL CHARGE TO PEER REVIEWERS

External Peer Review of U.S. EPA's draft *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity (Framework)*

Task Order No. 9
Contract No. EP-C-07-024
February 20, 2008

PRE-MEETING COMMENTS ARE DUE TO ERG BY CLOSE OF BUSINESS, Wednesday, March 26, 2008

CHARGE QUESTIONS

1. The purpose of the *Framework* is to provide an overall process by which chemicals can be evaluated and a determination made regarding whether the chemical has a mutagenic mode of action (MOA) for carcinogenicity. MOA is described as the key decision in risk assessment in U.S. EPA's 2005 *Guidelines for Carcinogen Risk Assessment (Cancer Guidelines)*, and the accompanying *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (Supplemental Guidance)* highlights the importance of ascertaining whether a chemical has a mutagenic MOA. The *Framework* document recognizes both the on-going research in this area, as well as the potential limitations of available data for making this determination.

    a. Please comment on whether the purpose for the document is clear.

The purpose could be more clearly stated. It appears to this reviewer that the basic purpose is to identify chemical compounds that (1) clearly act in vivo by directly damaging DNA in somatic cells and causing heritable somatic cell damage as a result and that (2) also are carcinogenic; and to link the DNA damage to the carcinogenicity in a convincing way. A second purpose is to identify carcinogens with properties that justify using the age-dependent adjustment factors developed by the EPA in its Supplemental Guidance for quantitative risk assessments. The weakness of the document is that it does not specifically acknowledge that there are other pathways, some of them indirect, by which xenobiotic chemicals can effect DNA damage that may be both mutagenic and carcinogenic. It is not clear what a risk assessor is to do with such substances.

    b. Does the document provide a useful framework for this determination?

Overall, the document does provide a useful framework, but it could usefully be reorganized and significantly shortened by removing the incessant citations of various EPA documents from the text to footnotes or endnotes, so as not to interrupt and confuse the narrative.

c. Is the document clear, complete and objective?

It is definitely not complete, because it does not address risk assessments for substances that cause heritable DNA damage and tumors by pathways not involving direct DNA reactivity.

d. Are major limitations and assumptions clearly defined?

Yes.

2. Given the inconsistency in the scientific community of genetic toxicologists regarding the definition of mutagenicity, the *Framework* (section 1.4) proposes an *operational* definition of "mutagenicity" for a very limited use, i.e., determination of a mutagenic MOA *for carcinogenicity* under the U.S. EPA *Cancer Guidelines* and *Supplemental Guidance*. Please discuss whether this definition is useful and appropriate, considering the limited application in this *Framework*.

The "definition" seems to be an incomplete fusion of the concepts of unrepaired DNA damage, which may be mutagenic, and mutagenicity per se as identified by various assays. The document would benefit by a careful re-writing which might include a rephrasing of its title. Known mutations of greatest importance in tumor pathogenesis are loss-of-function mutations of DNA sequences (missense and nonsense point mutations, and deletions) and chromosomal damage that likewise leads to loss of proper function. There is no discussion of this in the narrative.

3. Section 1.2 (uses of mutagenicity data) was added in coordination with and at the request of other Federal agencies that wished to distinguish their use of such data from the use in this *Framework*.
   a. Is this section clear and sufficiently complete to accomplish that goal? Please suggest recommendations to improve this section.
   b. If this section is not complete, what other specific uses of mutagenicity data for regulatory purposes might be addressed?

This section should be relegated to an appendix. It does not contribute to the purpose of the EPA Framework.

4. Which version of Figure 1 best captures the steps proposed in the *Framework*? Please identify the basis for your recommendation.

Version 2 is better; it gives more detail. The second part of this version needs to be integrated with the first part, however; at present it looks at first glance like a stand-alone table.

5. The MOA framework is defined in the *Cancer Guidelines.* The steps are repeated in the *Framework* with a description that more specifically discusses MOA as it applies to determining a mutagenic MOA for carcinogenicity.

   a. Is the information in this *Framework* on the application of the *Cancer Guidelines* MOA framework sufficiently clear and complete to be useful?

A few good examples would add greatly to the clarity of the presentation. The current version is verbose and the organization of the material is less than optimal. I suggest that the discussion of detecting mutations in various test systems (which is purely empirical and has nothing to do with the chemical properties of a test agent) might more appropriately follow the discussion of DNA damage and its detection. The issue is whether the analysis should follow the biological sequence of events (DNA damage → mutations → → → tumor), or its reverse since data are usually accumulated in the reverse order (carcinogenicity data in animals → mutation assays in vitro and in vivo → ADME and DNA reactivity data).

b. Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data that are generally available for consideration? If not, what additional elements might be useful to include in the *Framework*?

c. Please review each step, e.g., "key events," "dose-response relationships," "temporality," and discuss what types of additional information might be added (remembering that this document can not suggest or require additional testing). Given the current state of science, please comment on the application of each step in the MOA framework.

6. Is section 2.2 (Evaluate the Data against Current Acceptance and Quality Criteria) transparent to the reader? Does it adequately describe how the quality of the data can be evaluated? If not, how could it be expanded or improved? Please provide specific recommendations.

7. Are the discussions of aneuploidy adequate? If not, how should they be expanded? Please provide specific examples and detailed information regarding recommendations.

8. Referring to section 2.4 (Apply the MOA Framework), does knowledge of the type of mutational event, or knowledge of mutational spectra, contribute to weight of evidence (WOE) for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.

Both kinds of data – type(s) of mutational events, and mutational spectra, are useful. Mutational spectra are much more labor-intensive to establish and may be less generally applicable because of their greater scarcity.

9. Referring to section 2.4 (last paragraph), please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.

It is difficult to envision proceeding with a mutagenic MOA analysis without a very extensive database. How does one establish that an agent is DNA-reactive and damaging, for example, without specific data?

Section 2.4, page 23: "if tumors at different sites are induced by the same MOA, they may be analyzed together." This sentence needs amplification; the appropriate "different sites" might include similar tissues in different anatomic sites, e.g. urothelium in bladder, ureter, and renal pelvis; glial tissue in brain and spinal cord; lymphoid tissue at any site. These are basically the same sites that are acceptably grouped together when analyzing tumor data.

Section 2.4.1, final paragraph: The choice of cancer-related genes given as examples is unfortunate; *Rb* is rarely involved in the pathogenesis of experimental tumors. Most such data will come from animal tumor studies, and the genes most likely to be encountered as mutated in animal tissues should be the ones cited: *ras*, *p53.*

10. The WOE for determining an MOA for carcinogenicity is described and determined in the U.S. EPA *Cancer Guidelines*. This document provides a framework for organizing data, determining relevance of those data, and considering issues in a mutagenic MOA for cancer. Please discuss if we have achieved this goal. If not, what changes could be made in the Framework document to improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?

11. For groups of similar compounds, e.g., nitrosoamines or polycyclic aromatic hydrocarbons (PAHs), could a mutagenic MOA be supported solely by structure activity relationships or analogy? Apart from the consideration of groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by structure activity relationships or analogy? If not, what additional information would you think might be needed?

I do not endorse this concept. Structure-activity (S/A) relationships can be very misleading, because the structural element itself in a series of compounds (epoxides, N-nitroso groups) is often strongly influenced by adjacent parts of the molecule. Some epoxides, for example (e.g., d-limonene epoxide) and N-nitroso compounds with alkyl groups longer than about 5 carbon atoms are practically inert under biological conditions. Uncritical reliance on S/A relationships is likely to result in a large number of false positives.

12. Please comment on the discussion of the use of data in supporting a mutagenic mode of action for carcinogenesis in animals (Section 2.4.9) and in humans (Section 2.4.10).

The discussion of the importance of systemic mutagenesis is good. Perhaps too much emphasis is placed on identification of organ-specific mutations, as data are practically never available for some important tumor sites in rats and mice for purely technical reasons (small volume or inaccessibility of tissue from, e.g., Zymbal gland, forestomach, Harderian gland, mesothelium, thyroid, etc.)

13. Are the appendices of the document useful for the process of determining a mutagenic MOA for carcinogenicity? Please comment on how their usefulness and clarity may be improved.

Addition of a few well-researched examples, with both positive and negative conclusions, would be extremely useful in illustrating the application of the framework.

14. Please provide any other comments or concerns, both strengths and weaknesses, with the document not covered by the charge questions above.

The usefulness of the document is diluted overall by excessive and distracting references to the EPA Guidelines and other documents. It would be more useful if the document itself were shortened, the authorities that are cited were removed to footnotes or endnotes, and the key events were systematically presented in either the order in which data are most likely to accumulate (experimental tumors, mutagenicity in vitro & in vivo, ADME and DNA adduction) or the order in which the successive biological effects at the cellular and subcellular level must occur.

Toby Rossman

Comments on U.S. EPA's draft "Framework for Determining a Mutagenic Mode of Action for Carcinogenicity"

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Charge Question 1a

The purpose of the document is clear. The authors are to be congratulated on addressing this difficult issue.

Charge Question 1b

The usefulness of the framework is good as far as it goes, but does not adequately address 2 issues which I consider of extreme importance: 1) the issue of heritable mutations vs. premutagenic lesions; 2) the issue of cytotoxicity, especially in some in vitro assays.

By definition, the "mode of action" (MOA) of a carcinogen is "a sequence of key events and processes, starting with interaction of the agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation" (USEPA, 2005). For mutagenesis to be a carcinogenic MOA, the agent must cause heritable mutations (a redundancy that bears emphasis here) in cells that survive the treatment and are able to replicate and form mutant clones during the mutant selection protocol. This condition is satisfied by gene mutation assays that score mutant clones. It is not satisfied by many other "genotoxicity" assays that are commonly used (sometimes correctly, sometimes incorrectly) for hazard identification, such as chromosome aberrations, micronuclei, comet assays, DNA lesion measurements and DNA repair assays. These represent premutagenic events that may or may not result in heritable mutations.

Page 17, lines 16-18, mix together mutations and premutagenic lesions. WOE should have more of a hierarchy of importance.

Page 28, lines 15-35 also assumes that DNA damage in target organs necessarily leads to mutation. In fact, micronuclei often trigger apoptosis, and DNA strand breaks may be a sign of cytotoxicity. DNA adducts may be repaired. These tests would have greater power if measures of apoptosis and necrosis (e.g. by tissue staining) ruled out excessive cytotoxicity. It is also possible to carry out mutation assays in endogenous genes in animals (I believe that there are stains for hprt negative cells, once developed by Albertini).

Within the class of non-mutagenic assay results, not all represent pre-mutagenic DNA lesions (see page 20, bottom paragraph). DNA strand breaks can result from other mechanisms beside interaction of the chemical with DNA. SCE may reflect genomic instability rather than reactions with DNA.

Concerning cytotoxicity: Dead cells do not become tumors. To determine likely MOA, it is essential that accurate cell survival assessments be made. The gold standard is clonal survival, a method that is common in gene mutation assays. Short-term survival assays, such as MTT, neutral red, and trypan blue, as well as measurements of mitotic index that are commonly used in cytogenetic assays, fail to detect early apoptotic (or delayed apoptotic) events. Trypan blue detects necrosis. MTT and neutral red assays can be delayed to allow time for apoptosis to develop, at which point the results approach clonal survival (Komissarova et al., 2005).

In section 2.2, low cytotoxicity should be listed as a criterion of acceptance of data, especially for assays that do not depend on living cells for results.

Page 21, lines 14 on: The most common reason for conflicting results *in vivo* and *in vitro* have to do with different doses that can be tested. Many *in vitro* assays do not depend on viability for their results. Thus, unusually high doses that are not achievable *in vivo*, can give positive results *in vitro* that are driven by cytotoxicity.

Page 22, line 4, is one of few statements that considers cytotoxicity. Also, page 26, line 12.

Page 16, line 8: What is too high? Here is where cytotoxicity could be discussed.

Page 16, line 25: Biologically relevant needs more discussion. Here too, cytotoxicity may come into play (e.g. can a positive chromosomal aberration assay *in vitro* be attained at a dose that can be translated to *an in* vivo dose?)

Charge Question 1c

In general, the writing is clear. There are a few instances where the language could be sharpened. (e.g. page 25, line 7, should be reworded)

The Glossary should be placed at the beginning of the document. I didn't realize it was present until I reached section 4.

Pages 8-9 could benefit from further discussion of the differences between direct and indirect mutagenicity, as well as differences between heritable mutation and premutagenic lesions. Some of the problem seems to be with the *Framework* itself (e.g. p.9, lines 13-19)

In the strictest (and original) definition, genotoxic carcinogens damage DNA by covalently binding to it, either directly or after activation by metabolizing enzymes, or intercalate into the DNA-helix.

Indirect mutagenicity can be defined as interactions with non-DNA targets leading to mutagenic effects. It is expected that indirectly mutagenic agents should have a threshold concentration below which there is no effect, due to the fact that non-DNA targets exist in many copies in the cell, unlike DNA (Kirsch-Voldars et al, 2003a). (It is also the case that some directly mutagenic agents have a threshold, but that's a different matter). This distinction is not discussed in detail until page 22 (under Key Events). I have drawn up a list of events that can cause indirect mutagenicity:

Potential mechanisms for indirect mutagenicity

Interference with DNA repair
Interference with cell cycle control proteins
Interference with DNA replication (via interference with DNA metabolism or its precursors)
Interaction with nuclear proteins such as topoisomerases or spindle proteins
Nuclease release from lysosomes
Protein denaturation
Production of or change in reactive oxygen species
Interference with oxidative phosphorylation
Changes in ionic concentration, pH, or osmolarity

Charge Question 1d

Concerning major limitations and assumptions, the concepts of viability, heritability, and the differences between mutagenic and premutagenic lesions should be discussed more fully. This is discussed above.

Charge Question 2

Definition: A mutagen is an agent that can induce heritable changes in the DNA sequence of genes. Most direct mutagens do this by causing damage to DNA (pre-mutagenic lesions) that are converted to mutations after cell division via error-prone polymerases that can bypass unrepaired DNA damage. Some mutagens can act in other ways, such as base analogues (via misincorporation followed by mispairing), or various "indirect" mutagenic events, as outlined above.

Mutant cells must be able to generate a mutant clone. Assays that do not involve clonal growth should have far less weight than assays that do. The *in vitro* dose that causes an effect should be attainable *in vivo* without killing the host.

Charge Question 3

This would be OK, if the difference between Hazard Identification (HI) and MOA includes concepts of heritable mutation in living cells (for MOA, but not necessarily for HI).

Charge Question 4

The second figure is more complete, and I especially like the second page describing which results are more important. Still, the questions of heritable mutation, attainable dose (*in vivo*) and toxicity should be incorporated into the figure on the right-hand side between "Determine…." and "Sufficient…." boxes.

The list following Fig. 1 v2 is interesting and helpful. However, I feel that mutations should be given much more weight than primary DNA damage. They are linked together in 5 and 6. Gene mutations *in vivo* should be more important than DNA damage, since damage may or may not lead to mutation.

Charge Question 5a-5c

I believe that this question was already addressed above, by pointing out various topics that should be expanded, and suggested changes in importance of various assays. See comments above about pages 20-26.

Charge Question 6

I have mentioned the lack of discussion of cytotoxicity above. It applies to Current Acceptance and Quality Criteria.

Charge Question 7

Discussions of aneuploidy are not adequate. Especially in relation to the micronucleus (MN) assay.

In the past, several attempts have been made to distinguish between the aneugenic and clastogenic action of test compounds in the MN assay. Currently, the most widespread and reliable assays identify whole chromosomes in MN by labeling their kinetochores or centromeres. Kinetochore proteins can be identified by immunofluorescence with anti-kinetochore antibody (labeled MN are termed K+) while centromeric DNA sequences can be identified by FISH using repetitive DNA sequence probes (labeled MN are termed C+). However, only a few laboratories routinely use these techniques because they are very costly. When these techniques are used, the *in vitro* MN assay is considered a suitable alternative to *in vitro* chromosome aberrations tests for detection of clastogenic and aneugenic agents.

It is recommended that this assay should be performed under conditions of high survival (an increase of >90% in number of viable cells). It is also recommended that markers for apoptosis and necrosis be included (Kirsch-Voldars et al., 2003b). At least 2000 cells should be scored per concentration (1000 per culture, in duplicate).

A discussion of the threshold aspects of aneuploidy should be undertaken.

Charge Question 8

I have previously mentioned that the mutational event should be heritable. This presents a problem for chromosome aberrations (CA). In the 1990's attention was focused on CA as a result of extreme conditions such as high osmolarity, high ionic strength and low pH. These conditions, as well as some non-genotoxic chemicals (e.g. aphidicolin, an inhibitor of DNA polymerase □) caused interference with cellular functions that could lead to CA (Galloway et al., 1998). It was found that compounds that induced CA at <50% toxicity were more likely to be genotoxic in other assays than compounds that only caused CA at >50% toxicity. It is now recommended that chromosome aberration assays should incorporate some measure of cytotoxicity, although this is often not done adequately (Komissarova et al., 2004).

In vitro and in vivo assays should be carried out in conjunction with markers for apoptosis and necrosis. Clonal survival assays should also be used when using clonable cells. Care must be taken to apply these assays at the proper time, as we have found, with arsenite, that these markers often appear days after treatment (Komissarova et al., 2004).

Clastogenesis in situations of excessive toxicity may not be a realistic carcinogenic MOA. While traditional cytogenetic assays rely on short-term cell survival to generate the mitotic figures necessary for analyses, the long-term viability of these treated cells cannot be determined. It has been suggested that the increased number of mitotic figures, recorded in classic cytogenetic assays as mitotic index and used as indicators of "cell viability", may rather imply a cell cycle blockage at G2/M. Since cells with accumulating chromosomal aberrations at G2/M may not be viable in the long term, the relevance of this kind of data for carcinogenic risk assessment remains unclear.

A number of non-mutation events have sometimes been mentioned with regard to genotoxicity. These events may or may not lead to mutagenesis (as mentioned above). For example, the Comet assay detects both single and double strand DNA breaks as well as alkali-labile sites. Single strand breaks are quickly repaired and are not regarded as significant premutagenic lesions. Alkali-labile sites can result from some DNA adducts which spontaneously depurinate (or depyrimidinate) leaving AP sites which are cleaved by alkali. Also, base excision repair of adducts create AP sites and breaks as intermediates. Nucleotide excision repair also creates breaks.

DNA fragmentation into segments of 180 base pairs is a consequence of apoptosis, and apoptosis can also give a positive comet response even when induced by non-genotoxicants such as extremes of pH, ionic strength and osmolarity, and fas ligand (Henderson et al. 1998; Choucroun et al., 2001). Thus, it is necessary to evaluate apoptosis in the population of cells being used in the Comet assay. This is not usually done. One way to do this would be to evaluate the distribution of DNA damage by calculating tail moment in each cell. If apoptosis is occurring, a bimodal distribution will be seen with a population of cells that show no damage and a population of cells that show extensive damage. This occurs with compounds like dexamethasone and camptothecin (Lee et al., 2003). One can also use an Annexin V affinity assay or caspase-3 activation to detect apoptosis (Roser et al., 2001; Komissarova et al., 2004). When the Comet assay is performed in combination with an apoptotic assay, it has a higher specificity than the Comet assay alone (Lee et al., 2003). In order to avoid false positive responses, Henderson et al. (1998) suggest that the concentration of test substance should produce >75% viability. Necrotic cells also display DNA damage (Fairbairn et al., 1996).

Charge Question 9

This may be going too far.

Charge Question 10

I think this is answered above.

Charge Question 11

No, I don't believe that SAR is enough. You need real data.

Charge Question 12

I have commented above on my concerns that non-mutagenic (or possible pre-mutagenic) lesions are being substituted for mutagenic events in animal assays. I do believe that animal data of a truly mutagenic type can be informative about humans. Mutagenic carcinogens are more likely to induce tumors at multiple target sites and in multiple species compared with non-genotoxic carcinogens, so an animal mutagenic carcinogen is very likely to be a human mutagenic carcinogen.

Charge Question 13

Appendix A lacks a column for cytotoxicity!

Appendix B was informative on different batteries used by various agencies.

Appendix C was too sketchy to be of use. I would like to have seen examples of where SAR was helpful as well as where it failed.

Appendix D was not helpful. There are a number of papers that give numerical values as far as Sensitivity, Specificity, and Predictability of various assays. A Table could capture some of this information. Also, some chemical classes do better in some assays than others. The shortcomings of various assays could be discussed.

Charge Question 14: Misc. comments

The common view that carcinogenesis occurs primarily via direct acting genotoxic insults to DNA is too simplistic and does not fit the accumulating data for many human carcinogens. A challenge of risk assessment is to understand underlying indirect genotoxic mechanisms that may alter the presumptions made regarding thresholds (Lovell, 2000). In the European Union, considerations of indirect genotoxic mechanisms have led to new appreciation of thresholds in risk assessment (Pratt and Baron, 2003).

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Appendix D

List of Observers

Peer Review Meeting: EPA's Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity

Navy League Building
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Appendix E

Meeting Agenda

Peer Review Meeting: EPA's Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity

Navy League Building
2300 Wilson Boulevard
Arlington, VA
April 4, 2008

Agenda

8:00 a.m. **Registration**

8:30 a.m. **Welcome, Introductions, Meeting Purpose & Agenda**.. *Jan Connery, ERG*

8:40 a.m. **EPA Opening Remarks**
Lee Hofmann & Rita Schoeny, EPA Risk Assessment Forum

9:00 a.m. **Public Comment** ..... *Jan Connery*

9:30 a.m. **Discussion Process and Overarching Comments**
Bette Meek (Chair) & Panel

9:45 a.m. **Panel Discussion: Context and Definition of Mutagenicity**
Bette Meek & Panel
1a) Is the document's purpose clear?
2) Is the definition of mutagenicity useful and appropriate, considering the limited application in this *Framework*?

10:15 a.m. BREAK

10:30 a.m. **Panel Discussion: MOA Framework** ..... *Bette Meek & Panel*
1b) Does the document provide a useful framework for determining a mutagenic MOA for carcinogenicity?
5a) Is the information on the application of the *Framework* sufficiently clear and complete to be useful?
5b) Does the *Framework* provide an objective and transparent description of a process for determining a mutagenic MOA for carcinogenicity, given the types and amount of data generally available for consideration? If not, what additional elements might be useful to include?
5c) For each step, discuss its application (given the current state of science) and what types of additional information might be added (remembering that this document cannot suggest or require additional testing).
10) Does this document achieve the goal of providing a framework for organizing data, determining their relevance, and considering issues in a mutagenic MOA for cancer? If not, what changes could be made to

improve the evaluation of MOA information while remaining consistent with the *Cancer Guidelines*?

12) Sections 2.4.9 and 2.4.10: Please comment on the discussion of the use of data in supporting a MOA for carcinogenesis in animals and humans.

6) Section 2.2 (*Acceptance and Quality Criteria*): Is this section transparent to the reader? Does it adequately describe how data quality can be evaluated? If not, how could this section be expanded or improved?

8) Section 2.4: Does knowledge of the type of mutational event or the mutational spectra contribute to the WOE for a mutagenic mode of action? Please provide specific recommendations for using either the mutational event or mutational spectra.

7) Aneuploidy: Are the discussions of aneuploidy adequate? If not, how should they be expanded?

9) Section 2.4 (last paragraph): Please comment on the discussion (including examples) of proceeding with an evaluation of a mutagenic MOA for cancer when an extensive database is not available.

12:30 p.m. LUNCH

1:45 p.m. **Panel Discussion: Additional Questions** ..... *Bette Meek & Panel*

11) For groups of similar compounds, could a mutagenic MOA be supported solely by structure activity relationships (SAR) or analogy? Apart from groups of similar compounds, are there circumstances under which you would recommend that a mutagenic MOA could be supported solely by SAR or analogy? If not, what additional information might be needed?

3) Section 1.2: Is this section clear and sufficiently complete in distinguishing how the use of mutagenicity in the *Framework* differs from its use by other federal agencies? If not, how can this section be improved and what specific other uses of mutagenicity data for regulatory purposes might be addressed?

4) Which version of Figure 1 best captures the steps proposed in the Framework and why?

13) Are the appendices useful for determining a mutagenic MOA for carcinogenicity? How can their usefulness and clarity be improved?

2:45 p.m. **Panel Discussion: Concluding Comments** ..... *Bette Meek & Panel*

1c) Is the document clear, complete and objective?

1d) Are major limitations and assumptions clearly defined?

14) Does the panel have any other comments or concerns (both strengths and weaknesses) that have not already been discussed?

3:30 p.m. BREAK & **Writing Session**

4:15 p.m. **Development of Conclusions and Recommendations**. *Bette Meek & Panel*

5:25 p.m. **Closing Remarks** .....*Jan Connery & Lee Hofmann*

5:30 p.m. ADJOURN

Appendix F

Public Comment at the Meeting

James A. Swenberg, D.V.M, Ph.D

University of North Carolina at Chapel Hill

Comments on the "Framework for Determining a Mutagenic Mode of Action for Carcinogenicity"

James A. Swenberg, D.V.M., Ph.D

Kenan Distinguished Professor of Environmental Sciences and Engineering, Nutrition, and Pathology and Laboratory Medicine
Director, Center for Environmental Health and Susceptibility
Director, Curriculum in Toxicology

University of North Carolina at Chapel Hill

April 4, 2008

1. I am pleased to have the chance to provide comments to you today on the Draft "Framework for Determining a Mutagenic Mode of Action for Carcinogenicity." The document provides a clear opportunity to enhance the use of science in risk assessment. There is a strong scientific consensus that mutations represent the primary Key Events in the initiation and progression of cancer.

2. Mutations are heritable changes in the information content of a cell that can occur at the gene or chromosomal level. The "Framework" needs to clearly differentiate "mutations" from "genotoxicity," something the draft document does not do. Rather, it uses these two words as if they are interchangeable. They are not.

    a. DNA adducts come in many forms. They can be repaired, they may or may not be pro-mutagenic, they may or may not be chemically stable, they can arise from exposure to chemicals or their metabolites, but some also arise from endogenous processes. DNA adducts are not mutations, they are Biomarkers of Exposure.

    b. In contrast to DNA adducts, mutations are not repaired. The mutated cells can clonally expand or die. A mutation can be silent or change the transcription of proteins. Changes in proteins that lead to growth advantages, avoidance of cell death, invasion and metastasis are clearly associated with cancer.

3. DNA adducts represent Biomarkers of Exposure. As such, they provide a great deal of information on the molecular dose that informs our understanding of metabolism and repair. I have appended a recent publication that reviews these issues. It also describes the dose response for DNA and globin adducts, and demonstrates that at low doses they linearly decline towards zero over orders of magnitude.

4. In sharp contrast, mutations always have a background incidence that does not approach zero. Mutations that are induced by chemicals may have a linear or nonlinear dose response that extends down to background. When considering low dose extrapolation, Biomarkers of Exposure and mutations have different shapes or slopes. There are well understood reasons for this. Most exogenous DNA adducts only arise from a single chemical, so the molecular dose is directly related to the exposure. However, mutations arise from multiple sources, including exogenous chemical exposures and endogeneous DNA damage such as arises from reactive oxygen species, lipid peroxidation, DNA instability and polymerase errors. It is this background DNA damage that drives the biology that results in background mutations.

5. The "Draft Framework" never mentions these critical differences in dose response. In fact, there is no section that even discusses dose response. It is the dose response for mutations that should be utilized for low dose risk cancer assessment, not the dose response of Biomarkers of Exposure.

6. Most of the data on genotoxicity and mutagenicity have been collected for Hazard Identification i.e., under any conditions can it cause genotoxicity? To conduct a science-based Framework Analysis on a Mutagenic MOA, it will be necessary to examine the dose concordance of key events under conditions of the cancer bioassays.

Craig Barrow, Ph.D. DABT

The Dow Chemical Company,
On behalf of
American Chemistry Council

ACC's Perspectives on EPA's "Draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity"

Craig Barrow, Ph.D. DABT
The Dow Chemical Company
on behalf of
American Chemistry Council
April 2008

American Chemistry Council

Improving Agency Guidance to Keep Pace with Scientific Advancements

- EPA needs to ensure that progress is made towards updating Agency risk assessment guidance to:
  - reflect and incorporate the scientific community's greater understanding of the processes of chemical carcinogenesis
  - address the relevancy results in animal models to humans
  - promote more mechanistically-based risk assessments across EPA's programs.
- Guidance needed to provide consistency across the full spectrum of Agency programs and offices

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Improving Agency Guidance to Keep Pace with Scientific Advancements (cont.)

- Agency is to be commended for initiative to further utilize mode of action (MOA) in risk assessments
- Clearly, as reflected by the Agency, the ability of a chemical to "induce mutation in one of a number of mutation assays is not sufficient to conclude that it causes specific tumors by a MMOA or that mutation is the only key event in the pathway to tumor induction"
- But the draft guidance needs several specific improvements before being finalized

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Suggestions for Improving the Draft Guidance

- EPA needs to develop additional specific guidance that describes steps for:
  - organizing the available data
  - evaluating the relevance of those data
  - procedures for weighing all of the information and issues for a particular substance in determining a MMOA for cancer
- See Butterworth, Regul. Toxicol.Pharmacol. 45, 9-23, 2006)

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Suggestions for Improving the Draft Guidance (cont.)

Comments on two versions of Figure 1 (V1 and V2)

- V1 is more in line with the framework document and provides greater flexibility for applying the MOA framework than V2
- But, both V1 or V2 are lacking:
  - need to include chemicals that do not have mutagenic MOA for carcinogenicity (e.g., where direct DNA-reactive chemical induction of mutation is not an early key event in the process of carcinogenesis)
  - Need to distinguish chemicals for which mutagenicity is the key, and "driving" MOA versus those chemicals for which mutagenicity may play a minor role but in the absence of "co-MOAs" would not account for tumorigenesis

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Conclusions

- Agency is on the right path forward to develop guidance based on up to date scientific knowledge of MOA and carcinogenesis
- Agency needs to:
  - Develop case studies with actual datasets
  - Hold a workshop to apply the Draft MMOA Framework
  - This will help to define steps and procedures needed for interpreting actual results to determine a MMOA for cancer

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Conclusions

- Final MMOA Framework Guidance needs to have:
  - Specific and objective guidance for consideration of both individual studies and the full body of studies (weight of evidence)
  - Processes that result in a scientifically robust determination of whether a mutagenic mode of action is a non-factor, a minor factor, or a major contributor to the causal pathway of carcinogenesis by a specific agent under a specific set of exposure conditions

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Appendix G

Reviewer Post-Meeting Comments

Elaine Faustman

Post-Meeting Comments—Elaine M. Faustman

I would like to reiterate several points that I made in my pre-meeting comments and to urge all to review those as they are detailed and highlight both big picture as well as specific modifications/considerations that need to be addressed.

First, I feel that before the framework development can proceed, clarification of the definitions for mutagenicity is needed. It is not sufficient to acknowledge that the EPA has multiple definitions but resolution is needed otherwise great confusion will result as was evident even in the multiple comments and interpretation of the review team and external reviewers. This cannot be a narrow definition that does not address the full context of agency use. Nor should the definition create a new set of issues due to the fact that it does not fit with widely used definitions both internal to the agency nor external to the agency used by other national and international agencies. This would cause a tremendous amount of confusion.

Second, it is essential that a suite of examples be worked through the revised framework in order to ensure that the framework is constructed in a way that is useful, transparent and which provides value to an assessment. Numerous suggestions for both data rich and poor examples as well as examples with different types of genetic toxicity findings were given and should be run.

Third, it was unclear to the reviewers how different types of genetic information or mechanisms would be evaluated under this framework. This was true both for test results from hazard assessment types of assays as well as for more detailed assays looking at epigenetic responses. As written the framework would not be useful.

Forth, most importantly in this framework there is a reversal of the construct for burden of proof for showing that a chemical may work through a mutagenic mode of action. This reverses the previous frameworks and importantly violates one of the initial stated goals of the document that guidance in the two EPA reference documents will not be superseded. Of greatest public health concern is the switch in the underlying default assumption for the MOA framework. The proposed approach in the new framework would be to prove mutagenicity as a MOA rather than to disprove its relevancy as a mode of action. The definitions within the framework are not consistent with the approaches in either the current EPA guideline document for Carcinogen Risk Assessment (EPA, 2005) or supplemental guidance (EPA, 2005b) nor is it consistent with the International Agency on Carcinogens (IARC) Monographs or ICH testing paradigms. No on who is producing or using the agent would have an incentive to prove such a MOA and hence to require proof of mutagenicity shifts the burden of proof and incentives for data generation away from obtaining new scientific information. It also shifts the guidelines away from the use of lifestage protective factors based on this shift and not on a shift in scientific evidence or information.

Robert Heflich

Postmeeting comments on the EPA mutagenic MOA framework document prepared by Robert H. Heflich

This reviewer thanks the EPA and ERG for allowing me to participate in the review of the Framework. I have gained an appreciation of the extent to which EPA goes in considering all input from interested parties in formulating a document of this type, and the difficulties in accommodating all parties' views.

Some major messages that I would like to add for consideration and that were not necessarily clear in the transcript of the meeting:

1. Definition of 'mutagenicity'. I can appreciate EPA's desire to keep the definition of mutagenicity consistent between the various documents dealing with MOA. However, by the definition EPA uses, there are a number of compounds (i.e., mutagens) that produce mutations (common definition: heritable alterations in DNA sequence) by a process not involving what EPA calls mutagenicity. This is going to be a problem for many people to accept on its face. I know that EPA is a powerful agency, but co-opting a word with a commonly accepted meaning and redefining for its purposes with a 'working definition' may be a little difficult even for EPA to pull off. Perhaps using qualifiers with the word (e.g., the specific type of mutagenicity of importance to this Framework is the induction of mutation through DNA damage caused by the agent itself or the direct metabolites of the agent, referred to in the Framework as 'direct-damage mutagenicity' or something like that) would mitigate against the comments. Also, indicating up front what types of potential mutagenic events are excluded from your working definition of direct-damage mutagenicity (e.g., those caused secondarily as a result of oxidative damage, aneugenicity involving interaction with proteins) and why (because there are insufficient data linking these mutations to the initiation of tumors at the doses encountered in human exposures or something similar) might help acceptance. The Framework seems to be dividing mutations into two categories, those that it is concerned with (and wants to include in the working definition of mutagenicity) and those that it is not (and wants to exclude). Many people, however, will find this difficult to comprehend without a full clear explanation, and a repeated qualifier for the word in question.

2. Coming into the meeting, my reading of the Framework indicated to me that determining a mutagenic MOA is a linear process, with the process stopping with a negative conclusion at the second or third steps:

    a. Gather and evaluate the quality of all pertinent data (this not only includes mutagenicity and genotoxicity data, but data on carcinogenicity and data relating to other potential MOAs, see below);
    b. Determine whether or not the (rodent) carcinogen being evaluated is a mutagen (done mainly by a WOE evaluation of data from validated hazard ID assays) (if not mutagenic stop the mutagenic MOA evaluation and apply whatever frameworks exist for determining nonmutagenic MOAs);
    c. If the agent is both a (rodent) carcinogen and a mutagen, apply the modified Hill criteria to determine if the agent's mutagenicity is responsible for its

tumorigenicity, i.e, determine if the agent has a mutagenic MOA (if no, stop the mutagenic MOA evaluation and apply frameworks for determining nonmutagenic MOAs);

d. If the agent has a mutagenic MOA for animal tumors, determine whether or not the mutagenic MOA is relevant to human exposures.

This view of a linear process, however, was not shared by other reviewers, and I can see their point. It is probably better to proceed with the analysis as an iterative process, with later steps informing and altering some of the conclusions from preceding steps and with not aborting the process at intermediate steps. In addition, see the next comment.

3. Based on the discussions at the meeting, I also have come to the opinion that it may not be practical (or possible) to evaluate for a mutagenic MOA without using data that relate to other MOAs. That is because mutation may be a feature (even a key event but not the key event) of agents that act partially or mainly by nonmutagenic MOAs. What, for instance, if the primary effect of the agent was to disable DNA repair (e.g., by gene silencing through methylation) which then resulted in an increased mutant frequency. Or what if the agent acted as a mitogen and the increased cell division resulted in an increase in mutant frequency. Without applying the Hill criterion of temporality and appreciating that these nonmutagenic key events preceded and were responsible for the increases in mutant frequency (which may have been the proximate cause of the tumors), one might presume that the agent was solely acting through a mutagenic MOA. In fact, the mutations were induced secondarily to what might be considered the primary key event. Put in general terms, if the modified Hill criteria are used for evaluating all types of MOAs (as would seem to be indicated by the Cancer Guidelines), it may be more logical to consider all types of MOAs in a single analysis. In other words, if the modified Hill criteria are used for the mutagenic MOA evaluation, data will have to be gathered and considered for all possible MOAs anyway, so why not consider all possibilities in a single process? Perhaps work with test cases will shed some light on this.

4. The application of the modified Hill criteria, especially the criteria of dose-response concordance and temporality, to determining a mutagenic MOA would seem to demand the use of in vivo genetox data and data from nontraditional types of mutational analyses (at least nontraditional for the regulatory community). I don't think this is emphasized in clear enough terms in the Framework. Hazard ID data, which are discussed at great length in the Framework, only will be important to determining if an agent has mutagenic potential, and in partially fulfilling the first Hill criterion. As pointed out by our Chair, the real goal of the MOA analysis is tying the potential mutagenicity of an agent to the production of a specific tumor in a cancer bioassay. For that, I suspect, the criteria of dose-response concordance and temporality will be invaluable, and in vivo data will be necessary. The table that was part of the second version of Fig. 1 addressed some of the types of data that might be relevant to the process, but I think this table was misplaced in the document and not used properly to explain the process.

5. Discussions at the meeting made me realize that, when there are not enough data to establish whether or not a carcinogen is acting through a mutagenic MOA, there are

insufficient incentives for parties with an economic stake in the outcome of the risk assessment to provide additional research on the question. This is unfortunate and may result in the ADAFs being applied very infrequently.

6. To reemphasize this point, to establish a mutagenic MOA, it should not be enough to establish that a carcinogen is a mutagen, even if that mutagenicity is supported by a pile of hazard ID data. This amounts only to a high-order WOE evaluation of mutagenic potential. In this reviewer's opinion, the important part of a MOA evaluation is connecting the mutations induced by the agent with the specific tumors that it produces. This will require types of data not typically employed for mutagenic hazard ID. These types of data could include, but should not be limited to:

    a. An analysis of the temporality (and dose response) of in vivo mutation induction, along with the induction of cytotoxicity, apoptosis, compensatory cell proliferation, changes in gene expression, hormonal status (ect.) in comparison with the production of preneoplastic lesions and then tumors in the target tissues for tumors.
    b. Mutational spectra analysis of the mutations caused by the adducts formed by the agent in comparison with the mutations found in the oncogenes and tumor suppressor genes that are causative of the tumors;

    In practice, I doubt the perfect data set will ever be available, and how much data are necessary to make the connection of agent-induced mutations with tumors (I expect) will be determined by policy and the judgment of the risk assessors on a case-by-case basis.

Joseph Landolph

Post Meeting Comments from Dr. Joseph R. Landolph
May 8, 2008

After the meeting, I had the following suggestions for EPA on the Framework Document: Dr. Landolph's Personal Suggestions and Recommendations:

1. Regarding use of SAR in carcinogen regulation and in determining whether a chemical had a mutagenic MOA for carcinogenesis:

With polycyclic aromatic hydrocarbons (PAH), one can look at a molecule's structure, and determine whether a "Bay-Region" exists. If the molecule does have a "Bay-Region", then it is very likely to be a carcinogen. One can then conduct quantum mechanical calculations, and determine whether the formation of a "Bay-Region" diol epoxide would lead to a carbonium ion with significant resonance stabilization, resulting in a mutagenic metabolite. This thinking was worked out many years ago due to the efforts of Dr. Donald Jerina and colleagues at NIH. In this case, without data, one can come to some conclusion that a specific PAH would be likely to be a carcinogen. This would prioritize specific PAHs for mutagenicity testing. However, even in this best case, one should not rely on SAR alone, but rather use it to prioritize chemicals for testing, and then conduct rapid mutagenesis assays in bacteria and mammalian cells. I (and several other members of the committee) would not recommend relying solely on SAR.

If there was absolutely no experimental data available, and one had to regulate a carcinogenic class, this would work best for the PAH. However, even for this class of PAH, it would be dangerous from both an academic credibility viewpoint and also from the viewpoint of almost certain legal challenges, to rely only on SAR to determine whether a carcinogen had a mutagenic MOA. Here, one should use SAR to prioritize, and then conduct quick mutagenesis assays to obtain some real data before regulating carcinogens or determining whether or not a specific chemical carcinogen had a mutagenic MOA.

2. Regarding use of case studies in the Appendix of the Framework for Mutagenic MOA Document:

I recommend strongly that EPA first work up Aflatoxin B1 and Vinyl chloride, as case studies. These are known, strong, mutagenic, human carcinogens. They would provide a good set of first examples as to how to determine whether other chemical carcinogens act by a mutagenic MOA. The data on carcinogenesis and binding of these carcinogens to DNA to form carcinogen-DNA adducts is very strong, and these two events parallel each other, making it very clear that these two carcinogens act by a mutagenic MOA. If you work these two carcinogens up into case studies, they could serve as positive controls for the whole process. This would calibrate the process, and allow us to see where other carcinogens either met or fell short of data indicating that they acted through a mutagenic MOA for carcinogenesis.

Similarly, it would be very instructive to add a case study on a chemical that is almost certainly acting via a non-mutagenic mode of action to induce carcingoenesis. An example well-studied by EPA (Dr. Birnboim's Group) would be TCDD. This would let readers see exactly where you would depart the scheme in considering that a chemical carcinogen acted by a non-mutagenic MOA. It would also be instructive to consider a chemical that perturbs DNA methylation, such as 5-azacytidine, which should almost certainly act via a non-mutagenic MOA, through inhibition of DNA methylation, to cause carcingenesis. This chemical, or similar chemicals, could serve as a "gold standard" for chemicals causing cancer through a non-mutagenic MOA. Use of such positive and negative controls in case studies could help properly calibrate the process for determining whether a novel chemical acted through a mutagenic MOA or not to cause cancer. These case studies could be placed in the Appendix, and would be very worthwhile.

3. Regarding Mutagenicity vs. Genotoxicity in the Framework Document: I recommend that the EPA give some thought to dividing the world of carcinogens up as follows:

a. Genotoxic Carcinogens
   1. Mutagenic Carcinogens (defined broadly – DNA adductors, ROS-generating chemicals/radiations, agents that cause additions, deletions, frameshift mutations, gene amplifications, translocations, chromosomal aberrations; asbestos would probably fall in here).
   2. DNA-damaging but non-mutagenic carcinogens
      (Those that only cause DNA damage but not mutations later, those that cause SCEs, those that cause increased COMET tails, but no heritable mutations).

b. Non-Genotoxic Carcinogens
   1. Agents that perturb DNA methylation (5-azacytidine, and like agents).
   2. Agents that act through receptor occupancy to stimulate cell growth and division (Dioxin, estrogen, progesterone, testosterone, etc., peroxisome proliferators, etc.)

In this way, EPA would be very clear about the universe of genetic toxicology, and that this Framework was dealing with category a-1 above, and that anything that did not fit this category would go into another category, and be dealt with specifically under that category. This would make the Framework Document very clear. I strongly recommend such a division of the universe of carcinogens into these or similar categories in the very front of the document. At this point, then mutations and mutagenicity should be defined clearly and conventionally but somewhat broadly, so EPA does not have to go back and keep revising this document. In addition, genotoxicity should be defined as the property of a chemical or radiation to cause damage to DNA, broadly defined. Then, mutagenicity should be defined as a subset of genotoxicity, such that a chemical or radiation could cause heritable change in the sequence of DNA.

I recommend that you define it broadly as any heritable change in the sequence of DNA, including point mutations, deletions, additions, gene amplifications, mutagenic recombinational events, etc. I recommend that you stay with a generally accepted, but broad, definition of mutation for the Framework. This consideration would make the Framework Document for Mutagenic MOA most useful in the future.

I do not recommend any radical departures or simple operational definitions of mutagenicity. I also recommend against any "operational" definitions of mutagenicity. All scientists know what mutagenicity is. It is important to use conventional definitions of mutagenicity as a foundation upon which to build this document. This would make the terms very clear, and reduce the interminable arguments over definitions, which even occurred in our panel in trying to grapple with this document.

3. Regarding Figure 1 of the Appendix.

4) (Charge Question #4): Which version of Figure 1 best captures the steps proposed in the Framework, and why?

Regarding figures 1 and 2 in the Appendix, both versions are useful, but neither version is quite satisfactory at present. I personally liked Figure #2 better. I felt that Figure #2 (version #2) was more comprehensive, more complete, and clearer. However, it also requires some revision. I recommend using Version 2, and adding to it, for the middle path in the figure, "Data unavailable or insufficient to determine whether a chemical can induce mutation, or if a mutagenic chemical carcinogen has a mutagenic MOA." Then, please add another box, which says, "Mutagenic Chemical without a conclusive MOA for cancer; regulated by the default procedure as a carcinogen with a mutagenic MOA unless other non-mutagen MOAs can be substantiated." There is a high probability that for many chemicals, the data may be insufficient to determine whether an agent is mutagenic or not. Further, for many chemicals, there may be insufficient information to determine whether a mutagenic agent has a mutagenic MOA for a particular tumor or not. In addition, the questions of heritable mutations, attainable doses in vivo, and toxicity should be incorporated into the figure on the right side between "Determine" and Sufficient."

Therefore, I recommend choosing Figure 2, but additional work is needed on modifying figure #2 based on the above comments.

Regarding the Appendices:

The table in **Appendix A** would benefit from an additional column on whether or not the data conform to current guidelines for the assay. The repeated in vivo section should be removed, EPA should consider whether or not CAs and MNs are mutations. Appendix A is a useful way to organize data from genotoxicity/mutagenicity studies. Overall, the section is well-written and informative. We recommend adding a column to the in vitro studies table, encompassing DNA damage, as measured in the COMET assay and as measured by 8-hydroxy-deoxyguanosine formation in DNA caused by free radical formation. For the in vivo assays, I also recommend adding a row on measurement of 8-hydoxy-deoxyguanosine in DNA to measure DNA damage caused by free radicals.

Appendix B: This appendix provides a good abbreviated review of different mutagenicity testing schemes in use at EPA. Overall, this section was well-written and clearly written. We recommend incorporating a short discussion of the utility of assays to detect morphological and neoplastic cell transformation in cell culture. These assays detect many different types of chemicals, whether they act by mutagenic mechanisms (BaP), aneuploidization (DES), non-genotoxic/non-mutagenic mechanisms, or by combined mechanisms of action, including

genotoxicity (clastogenicity/gene amplification/micronucleus formation and also enhancement of methylation of tumor suppressor genes (insoluble nickel compounds). These tests have not realized their full carcinogen screening potential. They should be discussed briefly at the end of this section, and a few references added reviewing their utility. Here is an example of one review that should be cited to deal with this:

Landolph, J. R., Jr. Chemically Induced Morphological and Neoplastic Transformation in C3H/10T1/2 Cl 8 Mouse Embryo Cells, Chapter 9, p. 198-220, 2006. In, Molecular Carcinogenesis and the Molecular Biology of Human Cancer. Eds. David Warshawsky and Joseph R. Landolph, Jr. CRC Taylor and Francis Group, Boca Raton, Florida.

Appendix C: Examples of the Use of Structure-Activity Relationships in Assessing Mutagenicity. This section is very useful, is appropriately concise, is informative, and is well-written. The discussion of the different types of mutagenicity screening regimens accepted by different agencies was very interesting and informative for the reader. However, no mention is made of the efforts of the EPA and the NCI to use SAR to identify carcinogens based on chemical structure. This is increasingly important in this time of diminishing resources for genetic toxicology research and due to the large universe of chemicals that need to be studied for carcinogenicity (about 7,000 new chemicals per year and a backlog of approximately 100,000 total).

Appendix D: This section was written well overall. The section on Bone Marrow Chromosomal Aberrations/Micronucleus Induction was concisel and informative in discussing concordance between chromosomal aberrations/-micronucleus induction and carcinogenesis by ethylene oxide. The section on Studies on DNA adducts is concise and informative on the correlation between DNA adduct formation and carcinogenesis by various chemical carcinogens. The section on the Alkaline Single Cell Electrophoresis Assay (Comet Assay) was also informative and concise. I recommend adding a table to summarize these results, to break up the text and make the results more interesting. This is useful information. The section on In Vivo Transgenic Models is interesting, informative, contains useful data, and appropriately concise. A summary table of this data would also be interesting. This data showing a concordance between organ specific carcinogenesis and mutation in that same organ is excellent and very interesting data, and goes a long way toward establishing a mutagenic MOA for the specific chemicals mentioned in the text. The section on Use of Toxicogenomic Data is appropriately concise, informative, and appropriately conservatively written for this novel technology.

Bette Meek

Post Meeting Comments: M.E. Meek

The following comments were compiled from draft text developed by the panel at the April 4th meeting as a basis for potentially highlighting areas of agreement. It has become clear, however, that the draft text prepared and reviewed rather hastily during the session on April 4th is inadequate as a basis for outlining areas of agreement among panel members. This may well be a function of the lack of time available in the one day session to meaningfully consider areas of agreement in a controversial area in addition to responding to the significant numbers of questions of different levels of complexity posed to peer reviewers. These comments are offered, here, therefore, as post meeting comments of the Chair, alone. The limitations of the timeframe for the peer review to enable collective consideration of the large number of questions of uneven depth in a complex and controversial area should be noted. For this and other reasons, the input from the review might be best considered in the context of peer consultation to inform further refinement of a preliminary draft rather than peer review of a close to final product. Overall, the pre-meeting comments, which were developed independently by reviewers were in general agreement and much more detailed than those presented here. It is strongly recommended, therefore, that these comments be compiled and considered fully and carefully by EPA in revision of the Framework. Comments include specific suggestions to improve, particularly, the description of the analysis of MOA and associated human relevance (see comments from Meek, for example) and other areas identified below.

The purpose of the guidance should be more clearly and accurately stated and reflected in its organization and content. The basic purpose, as written, was to identify chemical compounds that (1) clearly act *in vivo* by directly damaging DNA in somatic cells and causing heritable somatic cell damage as a result and (2) also are carcinogenic.

However, for a "Draft Framework for Determining a Mutagenic Mode of Action (MOA) ***for Carcinogenicity,***" the focus should more appropriately relate specific DNA damage to a tumor-specific hypothesized MOA in a convincing way. Understanding of the MOA of induction of specific tumors, including contribution of heritable change in somatic cells (*i.e.,* mutation), may constitute an appropriate basis in some cases to justify using the age-dependent adjustment factors (ADAFs) developed by U.S. EPA in the Supplemental Guidance for quantitative risk assessments. However, the guidance included in the Framework in this area is unclear and inadequate, and does not appear to reflect the current state of experience and understanding of the broader scientific community related to consideration of the weight of evidence for hypothesized modes of induction of tumors including the role of mutagenicity and subsequent implications for dose-response.

Rather, the document focuses on screening of genotoxicity for the purposes of hazard identification for substances which may also induce tumors without meaningful integration of these two aspects. These screening assays are often conducted at rather high doses, including those that lead to significant cell death. In fact, they are designed to address a completely different question—yes/no in a screening hazard context to identify the need for additional testing versus dose-response for key events in a hypothesized MOA for tumors.

The lack of clarity about the application of genotoxicity screening assay results as a basis for prioritization for testing versus consideration of the weight of evidence for a hypothesized MOA for which mutagenicity may be a key event is additionally compounded by the content of Section 1.2, which describes the use of mutagenicity data by various parts of U.S. EPA.

More appropriate consideration of genotoxicity data, as recommended below, should not only increase our understanding of MOA for specific tumors, but also result in the development of testing paradigms

that more meaningfully address the relevant questions in risk assessment (*i.e.,* the nature of the dose response curve at more relevant doses).

Since the considerations of mutagenic MOA are specific for a tumor type and context, the title of the document should be revised to "Draft Framework for Determining ***Tumor-Specific*** Mutagenic Modes of Action (MOA)" to avoid misleading reference to mutagenic MOA in isolation of tissue type and context. It is also recommended that the text be reorganized to improve integration in an MOA context (see, for example, the suggestions in the pre-meeting comments from Meek, Heflich, and Rice). Reconsideration of the content in a MOA context also requires reframing particularly of content related to weighting of results of screening assays for genotoxicity in the context of a tissue specific MOA for tumors (i.e., consideration of a much broader array of more tissue-specific data for limiting key events for specific tumors which inform the subsequent dose-response analysis).

As a starting point to more appropriately frame the nature of data that informs MOA analyses for tumors, including a mutagenic component, the hierarchy presented in Figure 1 version 2, page 2 of the Framework is relevant. This version appropriately implies that the continuum of increasingly informative data becomes more precise and specific to the tumor type with the dose-response for key events more clearly informing quantitative relationships. In reframing the weighting of relevant data in the context of MOA for specific tumors, however, this reviewer suggests that the order be reversed and that (Quantitative) Structure Activity Relationship ([Q]SAR) be added to the first tier of the reordered hierarchy. This continuum provides guidance regarding the nature of test data that are helpful in considering MOAs of induction of specific tumors, including those with a mutagenic component, as a basis to better design relevant studies.

However, this reference to the data hierarchy presented in Figure 1, version 2 should not be taken to imply that either version of Figure 1 is satisfactory. Rather, it is recommended that a more appropriate figure be redrafted in the context of the comments presented here (*i.e.,* much better integration of data on mutagenicity in the context of MOA for specific tumors). The figure should describe the context-specific data for supporting or, most importantly, rejecting the hypothesized mutagenic MOA for a specific tumor type. The figure should also indicate the nature of additional data that would inform dose-response. A linear default for dose-response is currently applied in the absence of additional information.

The contribution of the results of (Q)SAR modeling to the weight of evidence of hypothesized modes of induction of tumors, including a mutagenic component, is necessarily dependent upon the purpose of any assessment, the nature of the (Q)SAR results (since SAR relationships for some classes of carcinogens (i.e., nitrosamines and PAH) are robust), and the extent to which the data contributes to the overall database. (Q)SAR contributes most to priority setting for additional testing, such as the conduct of screening assays for genotoxicity. (Q)SAR is also helpful in addressing disparate data sets in relation to mutagenic MOAs for specific tumors, but must necessarily be interpreted with a full understanding and appreciation of the basis on which predictions have been made and in the context of mode of induction of the specific tumors.

Decisions in the absence of additional data on mutagenicity beyond (Q)SAR modeling or screening assays for genotoxicity are necessarily context dependent, but must be health protective.

The data hierarchy within a tumor-specific mutagenic MOA framework additionally needs to consider implications for lifestage-specific data. In the absence of knowledge about the implications for lifestage based on an understanding of the MOA, a health-protective approach is advised, with a mutagenic MOA being assumed. This assumption would serve as a basis to encourage the generation of appropriate data. These considerations must take into account the context-specific mutagenic and non-mutagenic MOA mechanistic data.

Beyond these generic considerations of data relevant to mutagenic MOA, the pattern of integrated data determines relevant weighting. That the Framework does not consider the pattern and consistency of such data is a significant gap. Inclusion of case examples in the revised draft is essential to the provision of meaningful guidance in this context. Inclusion of case studies is also essential as a basis to inform users regarding the nature of critical missing data. Case studies should draw broadly on the continuing and evolving expertise in robust consideration of the weight of evidence for hypothesized MOAs for induction of tumors and their associated human relevance. To draw on and contribute to this expertise in the broader scientific community, the U.S. EPA should consider convening a workshop including both internal and external experts familiar with development of case studies in this area.

The content of the appendices also needs to be reconsidered in the context of their relation to assessment of the weight of evidence for hypothesized modes of induction of specific tumors with a mutagenic component. Inclusion of case studies with a framework analysis of the weight of evidence for hypothesized modes of induction of specific tumors including one or more mutagenic key events is preferred to best illustrate the more important aspects of data interpretation in an MOA context. A few well-researched examples, with both positive and negative conclusions, that illustrate various levels of data richness, the complexity of proposed MOAs, including multiple modes, and implications for lifestage are essential to meaningfully illustrate application of the Framework. The pre-meeting comments provide many excellent examples of potential and available case studies.

The proposed definition of mutagenic included in the document is also is currently too narrow and describes only one subset of all mutagenic events, namely those that cause direct damage to DNA (pre-mutagenic lesions) that is then converted after cell division via error-prone polymerases that can bypass unrepaired DNA damage. The definition inappropriately excludes indirect events that can lead to mutation relevant to tumor induction.

A preferred definition of mutagenic, which is consistent with the understanding of the broader scientific community and relevant to the appropriate focus of this document (i.e., consideration of mutagenic modes of action ***for tumours***), is a heritable change in mammalian somatic cells. This definition is necessarily relevant to hypothesized modes of induction of specific tumors, which is the appropriate focus of the Framework, and includes indirect pathways of xenobiotic chemical interactions with DNA that lead to mutation. These indirect pathways, some of which are known to be important in the genesis and evolution of certain human cancers, are extensively addressed in both the pre-meeting comments of peer reviewers (see p. 73, for example) and public comments (see Albertini and Walker, for example). Examples include the following.

- Heritable changes in somatic cells (*i.e.,* mutagenesis) can occur in many ways other than by direct interaction with DNA. These include base analogs (by misincorporation followed by mispairing) or various types of indirect mutagenic events that frequently result from reactions with proteins rather than DNA.

- Aneuploidy also is usually caused by protein damage or interference with the proper function of critical proteins or subcellular structures (*e.g.,* spindles). In their public comments, Albertini & Walker note that some aneuploidy-inducing agents may also directly damage DNA. Another mutagenic event, gene amplification (increased copy number) also may result from protein modifications such as mutant p53 protein.

- Oxidative damage/glutathione depletion, interference with DNA replication, etc. are also indirect modes of mutagenesis (see pre-meeting comments, p. 73).

Appropriate consideration within this framework of the implications for dose-response and life stage of the contribution of these types of mutational events to induction of specific tumors is critically important.

Moreover, some screening assays for genotoxicity do not measure heritable events (*i.e.,* mutation), but rather indicate evidence of DNA damage or its consequences. These include chromosome aberrations, micronuclei, comet assays, DNA lesion measurements, and DNA repair assays. Reference in the Framework to these assays in the context of mutagenicity is erroneous and inconsistent. Illustration by a Venn Diagram, with one circle representing genetic toxicity/DNA damage and another circle representing mutagenicity/mutation (a subset of genetic toxicity), might be helpful as a basis for illustration and consistent presentation throughout the document.

M.E. (Bette) Meek, May 12th/08

Toby Rossman

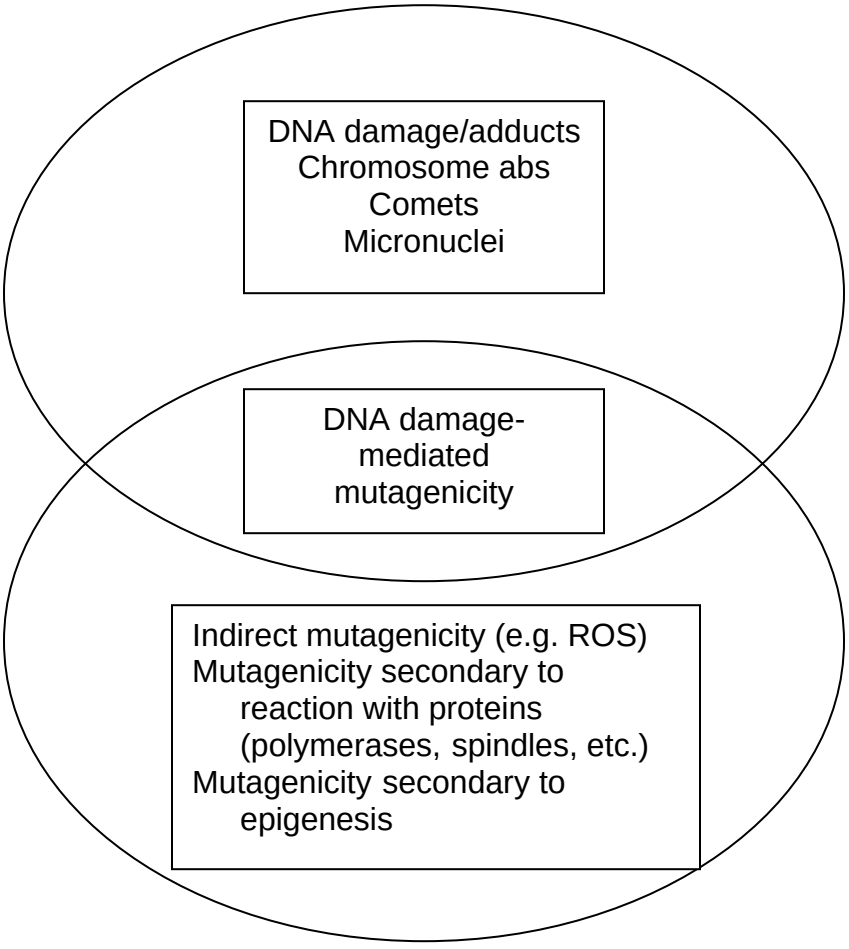
Post-meeting comment submitted by Toby Rossman

I. Purpose of Framework and Definition of Mutagenicity

The stated purpose of this document was to identify chemical carcinogens that clearly act in vivo by directly damaging DNA in somatic cells and causing mutations as a result. A second purpose was to identify carcinogens with properties that justify using the age-dependent adjustment factors developed by the EPA in its Supplemental Guidance for quantitative risk assessments. A weakness of the document is that it does not specifically acknowledge that there are other pathways, some of them indirect, by which xenobiotic chemicals can effect DNA damage or mutagenesis that may result in cancer. It is not clear what a risk assessor is to do with such substances.

The proposed definition of mutagenicity is too narrow. It describes only one subset of all mutagenic events, those resulting from DNA damage by the agent or its metabolite(s).

Genotoxic and Mutagenic Events



There are, however, mutagenic events that arise from events other than direct DNA damage by an agent or its metabolite(s). The Venn Diagram (which is not meant to be complete) illustrates some examples of this. Mutagenic events, in the context of a carcinogenic MOA, are simply those that cause heritable

change in mammalian somatic cells. **A mutagen therefore, as a more general definition, is an agent that can cause heritable changes in DNA sequences (order or amount).** Most mutagens do this by causing damage to DNA (pre-mutagenic lesions) that is converted after cell division via error-prone polymerases that can bypass unrepaired DNA damage (intersection of circles in Venn diagram). This was the only subset of agents that were specifically addressed in the mutagenic MOA document.

Mutagens that can act in other ways include base analogs (by mis-incorporation followed by mispairing), or various types of indirect mutagenic events that frequently are consequences of reactions with proteins rather than DNA. Aneuploidy also is usually caused by protein damage or interference with proper function of critical proteins or subcellular structures (e.g., spindles). Albertini & Walker (external public comments document) note that some aneuploidy-inducing agents ma also work via direct DNA damage. Another mutagenic event, gene amplification (increased copy number) also may result from protein modifications such as altered p53 activity. There are also several indirect modes of mutagenesis, including oxidative damage/glutathione depletion, interference with DNA replication, etc. (See pre-meeting comments, p. 73). None of these events is included in the definition of mutagenicity as used in this document, although some are known to be important in the genesis and evolution of certain human cancers. For these categories of agents, data are missing in the case of exposures of very young experimental animals compared to older ones. It is possible that risk from this kind of mutagenicity may also be appropriately adjusted using ADAFs and dose-response relationships for cancer risk assessment. Therefore, if the definition is not broad enough there is a chance of missing these other contributing tumor-specific mutagenic MOAs.

The document often lumped together genotoxicity assays that measure DNA damage with those that measure mutagenesis. Some assays that are used in genetic toxicology testing do not measure heritable events, but rather measure evidence of DNA damage or its consequences (Upper circle in Venn diagram). These include chromosome aberrations, micronuclei, comet assays, DNA lesion measurements, and DNA repair assays. They are generally used for hazard identification; however they are supportive evidence to a mutagenic MOA. They can be useful biomarkers of exposure.

For purposes of elucidating MOA, mutagenicity must be demonstrated at doses comparable to those that cause tumors in animals. Standard genotoxicity assays from hazard identification exercises thus usually cannot be used for purposes of establishing a mutagenic MOA, principally because doses used in such assays are too high and/or too toxic. However, somatic cell mutagenesis assays can be modified to study low-dose effects by using chronic exposure protocols such as those used to study spontaneous mutation rate.

II. Some Assumptions (mine) and Questions that need to be addressed (by EPA)

Assumption 1: This Framework is to be used with agents whose carcinogenicity has already been established.

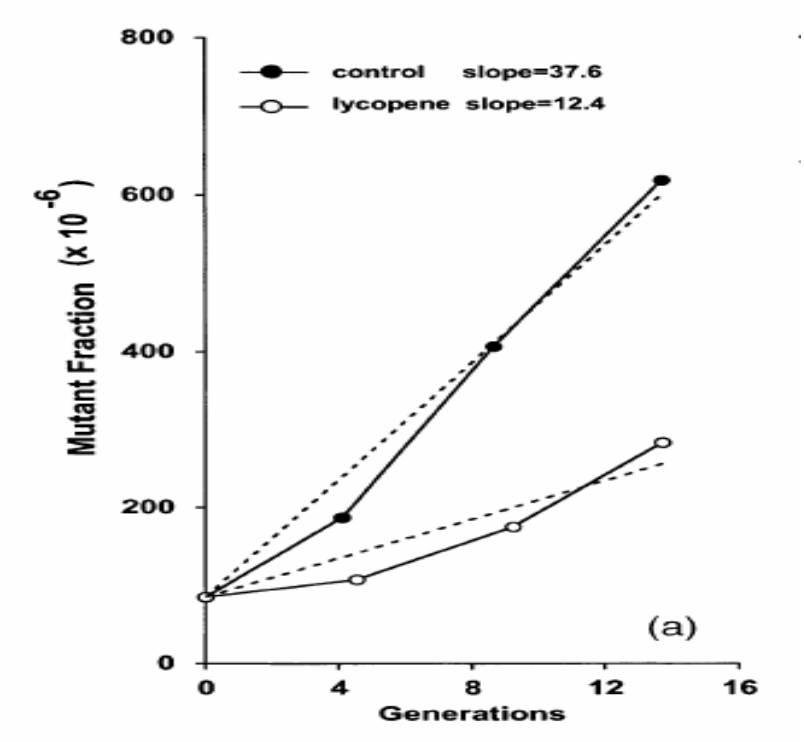
Questions:
Are we talking only about human carcinogens?
Does this require that human carcinogenicity data is sufficient (IARC Group 1)?
Does it also include probable (Group 2A)?
What about possible (Group 2B)?
Does this data have to be positive in a 2-year rodent bioassay to proceed?

Assumption 2: This Framework deals with agents that have already been adequately tested for mutagenicity (in somatic cells at endogenous loci) and yielded positive results for gene mutations. Agents (or metabolites) mutagenic in one type of somatic cell are generally also mutagenic in other cell types.

Agents that test negative for heritable mutation would be excluded from further evaluation for a mutagenic MOA since they are not mutagenic.

Questions: Should assays also be performed for heritable chromosome translocations and aneuploidy? How many and which types of assay systems are acceptable to exclude mutagenicity? Should mutagenicity be demonstrated in more than one cell type? Should human cells be used?

Assumption 3: A mutagenic MOA requires that Dose/Response relationships for mutagenicity should be consistent with biologically relevant doses to the target organ *in vivo*. It is not feasible at this time to establish such mutagenicity dose/response relationships *in vivo* in either humans or animals except for a few cell types (e.g. Albertini's work on human T-lymphocytes). Thus, somatic cells in culture must be used as surrogates. This requires that chronic low-dose studies of mutagenesis be carried out, similar to the approach developed in my laboratory for studying Endogenous (sometimes called Spontaneous) mutagenesis. Below is an example of how this assay was used to study antimutagens in mismatch repair-deficient human colon tumor cells that have elevated spontaneous mutagenesis (Mure and Rossman, 2001), but the same approach can be used to study very low concentrations of mutagens (which would increase rather than decrease the slope).



Antimutagenesis by lycopene in mismatch repair-deficient cells

Assumption 4: The dose to the target tissue (animal or human) must be evaluated in order to determine whether a mutagenic MOA (or any other MOA for which dose/response data can be obtained) is possible. Biomarkers of exposure become important here. Besides assays for adduct formation and other genotoxic endpoints, many new approaches can be considered (e.g. gene expression arrays, proteomics).

Assumption 5: In the absence of information on alternative MOA(s), a carcinogen whose mutagenicity can be demonstrated in the dose range that occurs in the target tissue at carcinogenic dose will default to a mutagenic MOA. This will encourage further research into possible alternative MOA(s).